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(54) Title: METHOD FOR DETECTION OF MUTATIONS

(57) Abstract

A method for detecting mutations, such as a single base change or an addition or deletion of about one to four base pairs, is based on the use of a DNA mismatch-binding protein, such as MutS, which binds to a nucleic acid hybrid having a single base mismatch or unpaired base or bases, thereby allowing the detection of mutations involving as little as one base change in a nucleotide sequence. Such a method is useful for diagnosing a variety of important disease states or susceptibilities, for example, detecting the presence of a mutated oncogen. The method shares the efficiency advantages of PCR-based methods but is much simpler and cheaper to perform. Also provided is a kit useful for practicing the method of the present invention.

The Principle of Mismatch (Mutation) Detection

METHOD FOR DETECTION OF MUTATIONS

Background Description

Assays capable of detecting the presence of a
particular nucleic acid molecule in a sample are of
5 substantial importance in the prediction and diagnosis of
disease, forensic medicine, epidemiology and public
health. Such assays can be used, for example, to detect
the presence of a mutant gene in an individual, allowing
determination of the probability that the individual will
10 suffer from a genetic disease. The ability to detect a
mutation has taken on increasing importance in early
detection of cancer or discovery of susceptibility to
cancer with the discovery that discrete mutations in
cellular oncogenes can result in activation of that
15 oncogene leading to the transformation of that cell into a
cancer cell (Nishimura, S. et al., Biochem. J. 243:313-327
(1987); Bos, J.L., Cancer Res. 49:4682-4689 (1989)).

Nucleic acid detection assays can be based on any of
a number of characteristics of a nucleic acid molecule,
20 such as its size, sequence, susceptibility to digestion by
restriction endonucleases, etc. The sensitivity of such
assays may be increased by altering the manner in which
detection is reported or signaled to the observer. Thus,
for example, assay sensitivity can be increased through
25 the use of detectably labeled reagents. A wide variety of
such labels have been used for this purpose, such as
enzyme labels (Kourilsky et al., U.S. Patent 4,581,333),
radioisotopic labels (Falkow et al., U.S. Patent
4,358,535; Berninger, U.S. Patent 4,446,236), fluorescent
30 labels (Albarella et al., EP 144914), chemical labels
(Sheldon III et al., U.S. Patent 4,582,789; Albarella et
al., U.S. Patent 4,563,417), modified bases (Miyoshi et
al., EP 119448), and the like.

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Several nucleic acid detection systems have been
designed to detect specific mutations, and involve a
combination of probes and additional binding partners
which act as reporters. Paau et al., U.S. Patent
5   4,556,643 discloses a polynucleotide probe having a
target-specific sequence and a protein-binding sequence.
In most cases, such a protein-binding sequence is an extra
segment of DNA ligated to the target-specific segment.
The binding proteins used can be part of a protein-marker
10 complex, such as a protein-enzyme complex (col. 7, lines
7-10). Disclosed binding protein were DNA-modifying
enzymes, polymerases, lac repressor, or antigenic DNA
sequences (col. 7, lines 10-29). A typical example of a
probe taught by this reference (Example I, col. 12 and 13)
15 is a cDNA molecule (capable of hybridizing to the target
DNA sequence) to which has been ligated T7 promoter (the
protein binding sequence). After hybridization of this
probe, an E. coli RNA polymerase is added as the binding
protein and binds to the T7 promoter. The presence of the
20 bound RNA polymerase is detected by a rabbit antiserum
specific for the polymerase followed by peroxidase-
conjugated goat-anti rabbit second antibody, and a
peroxidase-anti-peroxidase detection system.

Mundy, U.S. Patent 4,656,127, discloses methods for
25 detecting a mutation of a specific nucleotide base which
require knowledge of the sequence in the region of
interest (col. 1, lines 52-43), and have the advantage of
not requiring that the mutation be at a restriction site.
A linear probe complementary to part of the nucleic acid
30 chain is provided which extends in one direction from the
mutational site. Following hybridization, a nucleotide
derivative is added which joins the end of the probe
depending on whether a particular base sequence is present
or absent. The single stranded portion is digested. The
35 presence or absence of the probe is then detected (col. 2,

lines 3-22). The reference envisions a thionucleotide as
the nucleotide derivative (col. 4, lines 46-51), which is
detected by virtue of being detectably labeled.

Kato et al., European Patent Publication 407,789,
5 discloses methods for detecting a nucleic acid based on
hybridization with a labeled probe. Among the non-
radioactive labels mentioned are enzymes, and fluores-
cence, utilizing a biotin-avidin system (p. 2, lines 36-
39). The exemplified label is biotin-labeled dUTP which
10 is synthetically incorporated into the probe.

A number of methods designed to detect base-pairing
mismatches in nucleic acid hybrids is known in the art.
Earlier methods were based on enzymatic cleavage of
mismatches (Gibbs, R. et al., Science 236:303-305 (1987)).
15 These methods involve a step of enzymatically cleaving the
mismatched DNA hybrid. One drawback of these methods is
that they do not reliably detect all mismatches. A
recently described chemical method for cleavage of
mismatched DNA (Cotton, R.G. et al., Proc. Natl. Sci. USA
20 85:4397-4401 (1988); Cotton, R.G., Nuc. Acids Res.
17:4223-4233 (1989)) is based on chemical cleavage at a
mismatch site in DNA-DNA heteroduplex, using a number of
agents, in particular osmium tetroxide and hydroxylamine.
In this method DNA probes are prepared by restriction
25 enzyme cleavage of DNA of interest. Plasmid DNA
containing the sequence of interest is hybridized to
labeled probe DNA (either end-labeled or internally
labeled with ^{32}P). Hydroxylamine chemically modifies
mismatched cytosines; osmium tetroxide modifies mismatched
30 thymines. Piperidine is then used to cleave the DNA at
the modified sites, followed by polyacrylamide gel
electrophoresis (PAGE) and autoradiography to identify the
cleavage products. This method is said to have the
advantage of detecting all possible single base pair

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mismatches because, the method also results in cleavage at
a matched base pair in the vicinity of a mismatch.

Caskey and coworkers disclose a method for localizing
a mutation which utilizes PCR-amplified cDNA as a source
5 of template for the mismatch cleavage reaction. This
technique was applied in studying ornithine trans-
carbamoylase (OTCase) deficiency patients to map muta-
tions.

Kung et al., U.S. Patent 4,963,658, discloses
10 detecting single stranded DNA (ssDNA) by binding with a
high-affinity ssDNA-binding protein, such as a topo-
isomerase or a DNA unwinding protein (col. 2, lines 44-47)
which itself can be bound to a label, such as β-D-
galactosidase (col. 7, line 1).

15,   The desire to increase the utility and applicability
of presently available assays is often frustrated by assay
sensitivity as well as complexity and cost. Hence, it
would be highly desirable to develop more sensitive, as
well as simple and relatively inexpensive, assays for
20 detection of alterations in DNA.

Summary of the Invention
        The present invention relates to a method of
detecting mutations in a nucleic acid sequence which
involve as little as one base change in the sequence. The
25 extent to which a target polynucleotide occurs in a sample
can also be determined. The present method is based on
the use of a mismatch binding protein, as exemplified by
the MutS protein of E. coli and Salmonella. As used
herein, the term target polynucleotide is a nucleic acid
30 sequence which is to be detected. The target polynucleo-
tide sequence which does not contain the mutation (the
unaltered form) is referred to as the non-mutated target
polynucleotide or the non-mutated target sequence. The
target polynucleotide sequence containing a mutation or

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alteration is referred to as mutated target polynucleotide. The present method is useful for diagnosing a variety of important disease states or susceptibilities, such as, by detecting the presence of a
5 mutated oncogene. The method shares the efficiency advantages of PCR-based methods, but is simpler and cheaper to perform. The inventors have also devised a kit for carrying out the method of this invention.

The DNA being analyzed can be of any type such as DNA
10 obtained from blood cells, sperm or tissue of an organism, preferably a human. The method of this invention does not require elaborate or expensive apparatus, as is used in PCR, and can be carried out by a technician without sophisticated training. The method can be internally
15 controlled, such that the test sample and control DNA are subject to the same treatments. Finally the method can be designed to yield simple, easily interpretable results, such as a colored "plus" or "minus" sign on a membrane filter.

20 This invention is based on two key elements. The first is the existence of proteins which bind to a double stranded nucleic acid heteroduplex containing a mispaired or unpaired base. Well-known examples of such mismatch-binding proteins are bacterial proteins, such as the MutS
25 protein in E. coli, which function in DNA repair. The second element is the relative ease of isolating and sequencing specific genes and their mutant alleles.

Thus, the present invention is directed to a method for determining whether a mutation is present in the se-
30 quence of a single stranded polynucleotide (or target polynucleotide) in a sample. That is, it is a method of detecting a mutated target polynucleotide or, alternatively, of determining whether a mutation is present (or absent) in a polynucleotide (DNA or RNA).

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.In the method, a sample to be analyzed for the
mutated target polynucleotide is incubated with a single-
stranded polynucleotide hybridization partner which is at
least one single stranded base sequence complementary to
5   the sequence of the non-mutated target polynucleotide.
The hybridization partner and polynucleotides to be
assessed for the mutated target polynucleotide (e.g., a
DNA or RNA sample) are incubated under conditions suitable
to cause hybridization of the hybridization partner to any
10 mutated or non-mutated target polynucleotide which may be
present in the sample. Hybridization of complementary
sequences results in formation of a hybrid of the
hybridization partner and the target polynucleotide. The
hybrid formed is combined or contacted with a mismatch-
15 binding protein under conditions suitable to cause binding
of the mismatch-binding protein to mutated target polynu-
cleotide. This results in binding of mismatched binding
proteins to hybrids in which the target polynucleotide is
mutated target polynucleotide. The detection of the pres-
20 ence of the mismatch-binding protein bound to the hybrid
is indicative of the presence of a mutation in the
sequence of the polynucleotide of the sample (i.e., is
indicative of the presence of the mutated target
polynucleotide).
25   Preferably, in the above mentioned, the hybridization
partner is DNA, for example, cDNA or a synthetic
oligonucleotide. The target polynucleotide may be DNA or
RNA. A preferred RNA is mRNA.
         In the above method a preferred mismatch-binding
30 protein is the MutS protein or a functional derivative
thereof.
         In one embodiment of the method, the sample is a
biological fluid which has been subjected to conditions
sufficient to release and denature (render single
35 stranded) any nucleic acid present therein. Optionally,

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the released nucleic acid is subjected to a step of restriction endonuclease cleavage prior to the denaturation.

In one embodiment, the polynucleotide hybridization
5 partner is immobilized on a solid support and the resulting solid support-bound hybridization partner is incubated with a sample of polynucleotide to be assessed for the presence of mutated target polynucleotide. A preferred solid support is a nitrocellulose membrane.
10   The detection step preferably comprises adding a detectably labeled first binding partner which binds to the mismatch-binding protein. The first binding partner may be, for example, an antibody specific for the mismatch-binding protein or an enzyme which catalyzes
15 generation of a colored reaction product, which is detected.

In another embodiment, the detection step comprises adding a first binding partner which binds to the mismatch-binding protein and is not detectably labeled,
20 and a second binding partner which binds to the first binding partner and detects the presence of the second binding partner. The second binding partner may either itself be detectably labeled or a detectably labeled third binding partner which binds to the second binding partner
25 may be used. A preferred second binding partner is an antibody for the first binding partner. In another embodiment, the second binding partner is a MutL protein or a functional derivative thereof, preferably a fusion protein between MutL and a second protein or peptide
30 capable of being detected. A preferred second protein in the fusion protein is beta-galactosidase.

In another embodiment, the mismatch-binding protein is one component of a fusion protein, in which the second component is a second protein or peptide capable of being
35 detected, either directly (i.e, it is itself detectable)

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or indirectly (i.e., through the use of a reagent which is
detectable). In this embodiment, the detection step
preferably comprises adding a detectably labeled binding
partner which binds to the second protein or peptide
5   present in the fusion protein. The second protein may be
an enzyme capable of catalyzing the generation of a col-
ored reaction product from a chromogenic enzyme substrate.
The detection step comprises providing the chromogenic
substrate and visualizing the color reaction product. A
10   preferred second protein is beta-galactosidase.

        The present invention is further directed to a method
for detecting (distinguishing) a mutated from a non-
mutated sequence of a single stranded target mammalian
polynucleotide in a sample. In this method, a sample of
.15 mammalian polynucleotide (DNA or RNA) is obtained, using
known methods. For example, DNA or RNA can be obtained
from a cell, treated to render it single stranded and
incubated with a single-stranded polynucleotide
hybridization partner which is at least one single
20   stranded base sequence which is complementary to the
sequence of the non-mutated target mammalian poly-
nucleotide. The DNA or RNA and the hybridization partner
are incubated under conditions suitable to cause hybrid-
ization of the hybridization partner to any mutated or
25   non-mutated target polynucleotide which may be present in
the sample. Hybridization of complementary sequences
results in formation of a hybrid of the hybridization
partner and the target polynucleotide. The hybrid formed
is combined or contacted with a mismatch-binding protein
30   under conditions suitable to cause binding of the
mismatch-binding protein and hybrids, in which target
polynucleotide is mutated target polynucleotide.
The detection of the mismatch-binding protein bound to the
hybrid is indicative of the presence of a mutation in the
35   sequence of the mammalian polynucleotide of the sample

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(i.e., is indicative of the presence of the mutated target polynucleotides).

The present invention includes a kit useful for detecting a mutated target polynucleotide (distinguishing mutated target polynucleotide from non-mutated target polynucleotide sequence) in a sample. The kit includes the following:

5

(a)   a first container containing a single-stranded polynucleotide hybridization partner comprising at least one single stranded base sequence which is complementary to the non-mutated sequence of the target polynucleotide (i.e., is complementary to the non-mutated target polynucleotide);

10

15

(b)   a second container containing a mismatch-binding protein; and

(c)   a third container or a plurality of containers containing a reagent or reagents capable of detecting the binding of the mismatch-binding protein to the hybrid.

20

In the above kit, the mismatch-binding protein is preferably MutS or a functional derivative thereof. The reagent or reagents preferably comprise at least a detectably labeled first binding partner capable of binding to the mismatch-binding protein. A preferred first binding partner is an antibody specific for the mismatch-binding protein.

25

In an alternative kit embodiment, the reagent or reagents comprise a first binding partner which is not detectably labeled, the first binding partner being capable of binding to the mismatch-binding protein, and a second binding partner, such as an antibody, capable of binding to the first binding partner. The second binding partner may be detectably labeled. Also intended is a kit wherein the second binding partner is not labeled and a

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detectably labelled third binding partner capable of
binding to the second binding partner is included.

In a preferred kit embodiment, the first binding
partner is a MutL protein or a functional derivative
5   thereof, such as a fusion protein between MutL and a
second protein or peptide capable of being detected,
preferably β-galactosidase. A second binding partner is
capable of binding to the MutL protein or its fusion
partner protein, for example an antibody specific for β-
10   galactosidase.

In the above kit the hybridization partner is
preferably immobilized on a solid support. A preferred
solid support is a nitrocellulose membrane.

In another kit embodiment, the mismatch-binding
15   protein is a fusion protein fused with an enzyme and the
reagent comprises a chromogenic substrate for the enzyme.

Brief Description of the Drawings

Figure 1 is a schematic diagram showing the hy-
bridization of hybridization partners in the form of
20   oligonucleotide or cDNA hybridization partners with the
test or "target" DNA. The lower portion of the figure
shows the configuration of mismatch containing hybrids.
Where a cDNA has hybridized to a genomic DNA fragment,
non-hybridizing portions of the genomic DNA (introns) are
25   shown to loop out.

Figure 2 is a schematic representation of the
mutation detection assay of the present invention. In
this particular embodiment, the presence of the mismatch-
binding protein is detected through the use of a first
30   binding partner, an antibody specific for the mismatch-
binding protein. This antibody may be detectably labeled
or may be detected by a detectably labeled reagent (lower
right). Alternatively, the detection step can be

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amplified using a second binding partner, here an antibody
specific for the first antibody ("Anti-Ab") (lower left).

Figure 3 is a schematic diagram of a particular assay
format, as described in the Examples, wherein positive and
5 negative controls are deposited on a nitrocellulose filter
such that a visible "minus" sign is obtained if no
mismatch is present and a visible "plus" sign is obtained
if the target DNA contains a mutation (mismatch).

Figure 4 shows results of mismatch detection in
10 heteroduplexes annealed in solution.

Figure 5 shows results of mismatch detection in
heteroduplexes annealed to immobilized oligonucleotides.

Figure 6 shows results of mismatch detection in
heteroduplexes annealed in the presence of competing
15 oligonucleotides.

Description of the Preferred Embodiments

The present inventors conceived of a new, broadly
applicable and relatively simple method for detecting
alterations, even as a small as a single base change, in a
20 DNA sequence. Bacterial DNA mismatch repair mechanisms
employ a family of proteins, including a protein, which
recognizes and binds to double stranded DNA containing a
base pair mismatch (for reviews, see Radman, M. et al.,
Annu. Rev. Genet. 20:523-538 (1986); Radman, M. et al.,
25 Sci. Amer., August 1988, pp. 40-46; Modrich, P., J. Biol.
Chem. 264:6597-6600 (1989)). The MutS protein was
identified as such a component of the E. coli mismatch
repair system (Lahue, R.S. et al., Science 245:160-164
(1989); Jiricny, J. et al., Nucl. Acids Res. 16:7843-7853
30 (1988); Su, S.S. et al., J. Biol. Chem. 263:6829-6835
91988); Lahue, R.S. et al., Mutat. Res. 198:37-43 (1988);
Lahue, R.S. et al., Proc. Natl. Acad. Sci. USA 84:1482-
1486 (1987); Dohet, C. et al., Mol. Gen. Genet. 206:181-
184 (1987); Jones, M. et al., Genetics 115:605-610 (1987);

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Su, S.S. et al., Proc. Natl. Acad. Sci. USA 83:5057-5061
(1986); Dohet, C. et al., Proc. Natl. Acad. Sci. USA
82:503-505 (1985); Choy, H.E. et al., Mutat. Res. 142:93-
97 (1985); Jones, M. et al., Mol. Gen. Genet. 184:562-563

5   (1981)). Analogous proteins are known in other bacterial
species including MutS in Salmonella typhimurium (Lu, A.L.
et al., Genetics 118:593-600 (1988); Haber, L.T. et al.,
J. Bacteriol. 170:197-202 (1988); Pang, P.P. et al., J.
Bacteriol. 163:1007-1015 (1985)) and the hexA protein of

10 Streptococcus pneumoniae (Priebe, S.D. et al., J.
Bacteriol. 170:190-196 (1988); Haber et al., supra).

     Purified MutS protein binds DNA containing mispaired
bases, but does not bind DNA without mismatches or single-
stranded DNA. The MutS-DNA interaction does not result in

15 any degradation or modification of the DNA.

     This method is based upon detection of a mismatched
DNA heteroduplex formed when a strand of mutant DNA and a
"complementary" strand of wild-type DNA hybridize (see
Figure 1). The presence of the mismatch is detected in a

20 highly specific manner by first allowing a mismatch-
binding protein, such as the MutS protein E. coli, to bind
to the DNA duplex. The presence of the bound mismatch-
binding protein is then detected in any of a number of
ways, for example, by using a detectably labeled antibody

25 specific for the mismatch-binding protein, e.g., an anti-
MutS antibody. This method stands in stark contrast to
methods of the prior art which employ mismatch cutting
nuclease enzymes capable of breaking DNA at or near a
mispaired base pair. For a schematic illustration of this

30 approach, see Figure 2.

     The method of the present invention permits the
detection of the presence of a particular "target" nucleic
acid molecule or nucleic acid molecule or polynucleotide
in a sample. Such samples are typically biological

35 samples, such as, for example, blood, sperm, stool, sera,

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urine, saliva, culture medium, etc., as well as non-
biological samples, such as, for example, waste or
drinking water, milk or other foods, air, etc.

5   The target polynucleotide or nucleic acid molecule
detected by the method of the present invention may be
either DNA or RNA, but is preferably DNA. When the
hybridization partner is cDNA, the target nucleic acid may
be either DNA or mRNA, the mRNA being present in higher
copy number. As used herein, the term "target nucleic
10   acid", "target polynucleotide" or "target sequence" refers
to a nucleic acid which contains a sequence of interest to
be detected an/or quantified. It may include the wild
type sequence, in which case it is referred to as the wild
type or non-mutated target polynucleotide or non-mutated
15   target sequence. Alternatively, it may contain an
alteration or mutation (mutated from wild type), in which
case it is referred to as mutated target polynucleotide.
A target sequence in the target nucleic acid must be
sufficiently long to hybridize with a probe DNA
20   (hybridization partner) even if there is an alteration or
mutation within the polynucleotide. A target sequence
will preferably greater than 30 bases to be detected by
the present method. In one embodiment, the target
nucleotide sequence must be known, in order to permit
25   construction of a "hybridization partner" polynucleotide
or oligonucleotide having a sequence with the requisite
mismatch relative to the target sequence to permit the
binding of the mismatch-binding protein and its subsequent
detection.

30   In another embodiment, a cDNA molecule is used as the
hybridization partner. In this case, it is not necessary
to know in advance the target nucleotide sequence. Upon
hybridization of cDNA with a genomic DNA fragment, introns
not represented in the cDNA form unpaired loops (see
35   Figure 1), which are neither detected by the mismatch-

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binding protein nor interfere in its binding to single
base mismatches or to mismatches caused by addition or
deletion of 1-4 base pairs.

     The method of the present invention has the advantage
5 of permitting the detection of a mutant target nucleic
acid molecule (either DNA or RNA) without requiring the
amplification of such a molecule, as is common in a number
of more recently devised mutation detection assays. The
present invention accomplishes this goal through the
10 construction and use of one or more hybridization partner
oligonucleotide or polynucleotide molecules, including
cDNA molecules, having sequences complementary to one or
more wild-type target nucleotide sequences. The
hybridization partner is capable of hybridizing to a
15 target nucleic acid molecule, such that, when a base pair
mismatch or unpaired base occurs, this mismatch or
unpaired base is recognized by the mismatch-binding
protein.

     In a preferred embodiment, the hybridization partner
20 is a single-stranded nucleic acid molecule, most
preferably, single-stranded DNA.

     The target nucleic acid molecule may, for example, be
DNA or RNA from eukaryotic cells, prokaryotic cells,
viruses, viroids, or the like. Preferably, the target
25 nucleic acid molecule is of mammalian origin, most
preferably, of human origin. Indeed, there are no
constraints to the nucleotide sequence, or origin, of the
"target" nucleic acid molecule.

     Small frameshift mutations, either additions or
30 deletions, also result in a sequence which will pair with
the wild-type sequence in a manner such that the mismatch-
binding protein will bind. For example, it is known that
both the _E. coli_ MutS-based repair system (Radman, M. _et
al._, _Annu. Rev. Genet._ _20_:523-538 (1986)) and the
35 _Streptococcus pneumoniae_ Hex mismatch repair system act on

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mismatches resulting from the addition or deletion of a
single base. Additions or deletions of two or three base
pairs, and to a lesser extent of four base pairs are also
repaired, whereas an addition or deletion of five base
5   pairs is not. Thus, the methods of the present invention
are useful for detecting small addition or deletion
mutations of between one of four bases.

In an assay to detect a particular single base
mutation in double stranded (ds) DNA, it is preferable to
10 use two wild-type hybridization partner sequences which,
but for a single base, are complementary to each of the
two mutant DNA strands, since the sensitivity of the assay
will be increased by two-fold compared to the use of only
one hybridization partner. However, it is also possible
15 to use a single hybridization partner sequence
complementary to only one of the two DNA strands because
the assay is sufficiently sensitive such that the two-fold
decrease in sensitivity (compared to using two
hybridization partners) does not detract from the utility
20 of the method.

Because a mismatch-binding protein, for example MutS,
does not bind all possible mismatches with equal
efficiency (Dohet, C. et al., 1985, supra; Jones, M. et
al., 1987, supra), it may be necessary for certain target
25 sequences to prepare hybridization partners complementary
to both strands in the region of interest in order to
create both of the two possible mismatches from a given
dsDNA wild-type sequence in order to detect at least one
of the two mutant sequences.

30   The hybridization partner can be a single continuous
polynucleotide segment, complementary to a larger target
sequence wherein more than a single base pair is
mismatched. Alternatively, two mutations on a single
fragment of target DNA that are separated by about 30 or
35 more bases can be detected by using two or more individual

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hybridization partners, each of which hybridizes with a
separate sequence on the single target fragment.

In one embodiment, the region of the hybridization
partner complementary to the target sequence can be
5   flanked at the 3' or 5' termini by non-hybridizable
sequences, provided that the hybridizable portion forms a
stable heteroduplex of at least about 30 nucleotides
wherein the mismatch-binding protein can recognize a
mismatch and bind.
10   The RNA or DNA hybridization partner can be prepared
by a variety of well-known means. Most preferably, an
oligonucleotide hybridization partner is prepared by
chemical oligonucleotide synthesis using conventional
methods. Techniques for synthesizing oligonucleotides are
15 disclosed, for example, by Wu, R., _et al._, _Prog. Nucl._
Acid. Res. Molec. Biol. _21_:101-141 (1978)).
Alternatively, an RNA hybridization partner can be
isolated as a natural product of cells, for example, as an
mRNA molecule. RNA or cDNA hybridization partners can
20 also be prepared by recombinant DNA methods. (See for
example, Green _et al._, _Cell_ _32_:681 (1983)). DNA
hybridization partners can be prepared from any of a
variety of sources readily apparent to one of skill in the
art, for example by restriction endonuclease cleavage or
25 chemical cleavage of naturally occurring DNA.

Given the fact the _E. coli_ MutS binds to hetero-
duplexes of 16 bases (Jiricny _et al._, _supra_), and that the
size of the heteroduplex protected by MutS in a
footprinting assay is as little as 8-12 bases (Su, S-S. _et_
30 _al._, _Proc. Natl. Acad. Sci. USA_ _83_:5057-5061 (1986)), the
lower limit for the size of the hybridization partner of
the present invention is about 10 nucleotides. To
increase fidelity of hybridization while trying to keep
synthetic costs down, a larger hybridization partner is
35 preferred, preferably from about 20 to about 100

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nucleotides. Given the economics of oligonucleotide
synthesis, a more preferred oligonucleotide hybridization
partner will have from about 20 to about 40 nucleotides.
There is no upper limit on the size of the hybridization
5   partner, since recombinant methods can be used for its
production, as described herein. Thus, in another
embodiment, an intact cDNA molecule is the hybridization
partner.

For use in the methods of the present invention, the
10 hybridization partner sequence is in immobilizable form
or, more preferably, is immobilized on a solid phase
support or carrier (see Figure 3). An immobilizable form
of the hybridization partner will be one in which can be
conveniently rendered immobilized, generally subsequent to
15 the hybridization reaction. The means by which the
hybridization partner is ultimately immobilized is not
critical to the present invention, and any available
approach can be taken as long as the hybrids formed
between the hybridization partner and the target nucleic
20 acid sequence are rendered immobilized through a property
of the hybridization partner.

When introduction into the hybridization reaction in
an immobilized form, the hybridization partner can be in
any appropriate form that enables it, and any components
25 of the reaction mixture that have become bound to it, to
be subsequently isolated or separated from the remaining
mixture. Separation may be accomplished by
centrifugation, filtration, chromatography, decanting or
the like.

30   One of ordinary skill in the art will appreciate the
variety of compositions and configurations of an
immobilized hybridization partner useful according to the
present invention. For example, the hybridization partner
can be aggregated or otherwise precipitated, attached to
35 an insoluble material, polymer or support, or entrapped in

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a gel such as agarose or polyacrylamide. (Meth. Enzymol.,
12B:635 (1968); Proc. Natl. Acad. Sci. USA 67:807 (1970)).
Most preferred is a solid support to which the
hybridization partner is attached or fixed by covalent or
5   noncovalent bonds. Preferably, noncovalent attachment is
by adsorption using methods that provide for a suitably
stable and strong attachment. This can also be
accomplished by means of an amino modifier on the
hybridization partner; the amino modifier is used to
10 "hook" or attach the hybridization partner to a solid
support (e.g., a membrane or filter). The means for
attaching a desired oligonucleotide or polynucleotide to a
selected solid support are well known to those of ordinary
skill in the art.

15   By "solid phase support" is intended any support
capable of binding an oligo- or polynucleotide. Well-
known supports or carriers include natural and modified
celluloses, such as nitrocellulose, polystyrene, poly-
propylene, polyethylene, dextran, nylon, polyacrylamides,
20 and agaroses. The support material may be virtually any
possible structural configuration, as long as the
immobilized hybridization partner can hybridize to the
target nucleic acid molecule and the mismatch-binding
protein can subsequently bind to the hybridization
25 partner-target polynucleotide hybrid. Thus, the support
configuration can include microparticles, beads, porous
and impermeable strips and membranes, the interior surface
of a reaction vessel such as test tubes and microtiter
plates, and the like. Preferred supports include
30 nitrocellulose disks or strips. Those skilled in the art
will know many other suitable carriers for binding the
oligo- or polynucleotide hybridization partners, or will
be able to ascertain these by routine experimentation.
      A preferred method for adsorbing the hybridization
35 partner onto nitrocellulose membranes involves saturating

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a solution of the hybridization partner with sodium iodide and spotting or filtering aliquots onto the membrane (Bresser et al., DNA 2:243 (1983)). Alternatively, the hybridization partner can be treated with glyoxal (less

5   than 1 M), and then adsorbed to the membrane. The hybridization partner may be fixed by baking at around 80°C under vacuum for about 2-3 hours (Thomas, P.S., Meth. Enzymol. 100:255).

    Covalent immobilization of the oligonucleotide
10 hybridization partners can also be accomplished. Numerous support materials and coupling methods useful in covalent immobilization are known. Examples include coupling to phosphocellulose through phosphate groups activated by carbodiimide or carbonyldiimidazole (Bautz, E.K.F. et al.,
15 Proc. Natl. Acad. Sci. USA 48:400-408 (1963); Shih, T.Y. et al., Biochemistry 113:3411-3418 (1974)), or reactions of diazo groups on m-diazobenzoyloxymethyl cellulose with G and T residues of the hybridization partner (Noyes, B.E. et al., Cell 5:301-310 (1975); Reiser, J. et al., Biochem.
20 Biophys. Res. Comm. 85:1104-1112 (1978)). Polysaccharide supports can be coupled through phosphodiester links formed between terminal phosphate of the oligonucleotide and hydroxyl moieties of the support by water soluble carbodiimide activation (Richwood, D., Biochim. Biophys.
25 Acta 269:47-50 (1972)), or by coupling nucleophilic sites on the oligonucleotide with a cyanogen bromide-activated support (Arndt-Jovin, D.J. et al., Eur. J. Biochem. 54:411-418 (1975)). Further, 3' hydroxyl terminus of the hybridization partner can be oxidized by periodate and
30 coupled by Schiff base formation with supports bearing amine or hydrazide groups (Gilham, P.T., Method. Enzymol. 21:191-197 (1971); Hansske, H.D., et al., Method. Enzymol. 59:172-181 (1979)). Supports having nucleophilic sites can be reacted with cyanuric chloride and then with the

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polynucleotide (Hunger, H.D. et al., Biochim. Biophys.
Acta 653:344-349 (1981)).

In general, any method can be employed for immobi-
lizing the hybridization partner, provided that a sequence
5   complementary to the target sequence is available for
hybridization to a sample nucleic acid. Particular
methods or materials are not critical to the present
invention.

An alternative to employing directly immobilized
10 hybridization partner is to use an immobilizable form,
allowing performance of the hybridization step in solution
where the kinetics are more rapid. Typically, in such an
embodiment, one would use a hybridization partner
comprising a reactive site capable of forming a stable
15 covalent or noncovalent bond with a reaction partner and
obtain immobilization by exposure to an immobilized form
of the reaction partner. Preferably, such reactive site
in the hybridization partner is a binding site such as a
biotin or hapten moiety which is capable of specific
20 noncovalent binding with a binding substance such as
avidin or an antibody which serves as the immobilized
reaction partner.

Essentially any pair of substances can comprise the
reactive site/reactive partner pair which exhibit an
25 appropriate affinity for interacting to form a stable
bond, that is a linking or coupling between the two which
remains substantially intact during the subsequent assay
steps, principally the separation and detection steps.
The reactive site on the hybridization partner is referred
30 to as a "binding site" and the reaction partner is
referred to as a "binding substance" with which it forms a
covalent or noncovalent bond or linkage.

In one embodiment, the binding site is preferably
present in a nonhybridizable portion of the hybridization
35 partner and is preferably a result of a chemical

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modification of the hybridization partner. Examples of
binding sites existing within the nucleotide sequence are
a promoter sequence which can be bound by a promoter
protein, an operator sequence which can be bound by a
5   repressor protein, or a modified nucleotide which can be
bound by a specific antibody, for example, 5-bromodeoxy-
uridine or 5-iododeoxyuridine (British Pat. Spec.
2,125,964), thymine glycol (Rajagopalan et al., Radiat.
Res. 97:499-510 (1984); Hubbard, K. et al., Radiat. Res.
10 118:257-268 (1989)).

Binding sites introduced by chemical modification of
the oligonucleotide hybridization partner are particularly
useful and normally involve linking one member of a
specific binding pair to the hybridization partner.
15 Useful binding pairs from which to choose include
biotin/avidin (including egg white avidin and
streptavidin), haptens (or antigens)/antibodies, carbo-
hydrates/lectins, enzymes/inhibitors, and the like. Where
the binding pair consists of a protein member and a
20 nonprotein member, it will normally be preferred to link
the nonprotein member to the hybridization partner since
the protein member may be unstable under the denaturing
conditions of hybridization. Preferable systems involve
linking the hybridization partner to biotin or a hapten
25 and employing immobilized avidin or an anti-hapten
antibody reagent, respectively.

When the hybridization partner is introduced into the
hybridization reaction in an immobilizable form, the
subsequent steps of immobilization of the formed duplexes
30 and addition of the mismatch-binding protein and the
reagents for detection can proceed in any desired order.
Immobilization and addition of the mismatch-binding
protein and other reagents can be accomplished by
simultaneous addition of the necessary reagents and
35 materials, by sequential addition, with or without

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intervening washing or separation steps, in either order. Where ordered additions are followed, of course one will take into account the concentrations of the added reagents so as not to oversaturate the formed hybrids and inhibit
5   interaction with the mismatch-binding protein and the detection reagents.

       A preferred mismatch-binding protein is characterized by its ability to bind the DNA-DNA (or DNA-RNA or RNA-RNA) base pair mismatched hybrids formed between the
10 hybridization partner and target complementary sample nucleic acids, to the significant exclusion of the single stranded polynucleotides or perfectly matched hybrids. In a preferred embodiment, the intact native MutS protein from E. coli is used. However, as used herein, the term
15 "mismatch binding protein" is intended to encompass a functional derivative of the intact, native protein. By "functional derivative" is meant a "fragment", "variant", "analog", or "chemical derivative" of the protein which retains the ability to bind to a mismatch nucleic acid
20 heteroduplex, in accordance with the present invention.

       A "fragment" of a mismatch-binding protein refers to any subset of the molecule, that is, a shorter peptide. A "variant" of the protein refers to a molecule substantially similar to either the entire protein or a
25 DNA-hybrid-binding fragment thereof. A variant of mismatch-binding protein, for example, of MutS, may be prepared by recombinant DNA methods well-known in the art.

       A preferred functional derivative of MutS is a homologue of E. coli MutS in another bacterial species,
30 such as the MutS protein of Salmonella typhimurium (Lu, A.L. et al., supra; Habet L.T. et al., supra; Pang, P.P. et al., supra) or the hexA protein of Streptococcus pneumoniae (Priebe S.D. et al., supra; Haber et al., supra). In addition, possible eukaryotic homologues of
35 MutS or HexA can also be used, such as those encoded by

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the homologous sequences identified in human, mouse or
hamster DNA (Shimada, T. et al., J. Biol. Chem. 264:20171
(1989); Linton, J. et al., Molec. Cell. Biol. 7:3058-3072
(1989); Fuji, H. et al., J. Biol. Chem. 264:10057 (1989)).

5   A "chemical derivative" of the mismatch binding
protein contains additional chemical moieties not normally
a part of the protein, including additional stretches of
amino acids as in a fusion protein. Covalent
modifications of the peptide are included within the scope
10 of this invention. Such modifications may be introduced
into the molecule by reacting targeted amino acid residues
of the protein with an organic derivatizing agent that is
capable of reacting with selected side chains or terminal
residues. Examples of such derivatization is hapten-
15 modification, wherein a haptenic group such as
trinitrophenyl and the like is conjugated to the protein
and serves as the target of a detectably labeled binding
partner for detection purposes. Alternatively, the pro-
tein can be modified by conjugation of biotin groups, and
20 detectably labeled avidin or streptavidin can be used as
the binding partner for detection purposes.

         A preferred chemical derivative of the mismatch-
binding protein is a fusion protein between the mismatch-
binding protein and another protein (the "fusion protein
25 partner"), wherein the other protein provides a structural
or functional feature which makes it useful for detection
of binding. For example, the fusion protein partner may
contribute antigenic sites to which an antibody can bind
for detection purposes. A fusion protein between the
30 mismatch-binding protein or functional derivative and an
enzyme can provide a more direct means of detection, for
example, by virtue of the ability of the enzyme portion of
the fusion protein to catalyze the formation of a colored
reaction product from a chromogenic substrate, as is well-
35 known in the art.

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A preferred fusion protein is one between MutS or a
functional derivative and the enzyme β-galactosidase.   In
this embodiment, the presence of the β bound to the solid
support can be detected by using a chromogenic substrate
5 of this enzyme.   An example of such a substrate is NABG
(Naphthol AS-B1-β-d-galactopyranoside).

A fusion protein derivative of the mismatch-binding
protein can be prepared using methods well-known in the
art (see, for example, Sambrook, J. et al., Molecular
10 Cloning: A Laboratory Manual, 2nd Edition, Cold Spring
Harbor Press, Cold Spring Harbor, NY, 1989).

Selection of a protein as being useful for the
methods of the present assay can be performed by one of
ordinary skill in the art using conventional methods.
15 Thus, for example, in evaluating a source for the presence
of a mismatch-binding protein useful in the present
invention, one can perform a mismatch-binding assay, such
as that described for MutS by Jiricny et al. (which
reference is hereby incorporated by reference in its
20 entirety).   Preferably, a filter binding assay is used.
To prepare the oligonucleotide heteroduplex, an
oligonucleotide, preferably of about 16 bases is labeled
with ^{32}P using a kinase reaction and gamma-^{32}P-ATP using a
kinase such as T4-polynucleotide kinase.   The 5'-labeled
25 oligonucleotide (which can be stored at -20°C) is then
annealed with a complementary oligonucleotide having a
single base pair mismatch under standard conditions.   The
annealed 16 base pair heteroduplex is mixed with an excess
of the protein being tested and kept on ice for 30
30 minutes.   The mixture is then applied to a nitrocellulose
filter which has been prewetted in the assay buffer.
Gentle suction is applied for several seconds, and the
filter is washed extensively with ice-cold assay buffer.
The filter is then dried in the air, suspended in scintil-
35 lation fluid and counted.   By virtue of the protein

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sticking to the filter, any counts on the filter can be attributed to binding to the putative mismatch-binding protein. In the absence of such a protein, the labeled oligonucleotide heteroduplex will pass through the filter.
5 Thus, by using such a simple assay, one can easily detect and select a mismatch-binding protein useful in the methods of the present invention.

As is known in the art, various hybridization conditions can be employed in the method of the present
10 invention. Typically hybridization will proceed at slightly elevated temperatures, e.g., between about 35°C and 75°C and usually around 65°C, in a solution comprising buffer at pH between about 6 and 8 and with appropriate ionic strength (e.g., 2X SSC where 1X SSC = 0.15 M sodium
15 chloride and 0.015 M sodium citrate, pH 7.0), protein such as bovine serum albumin, Ficoll (a trademark identifying a copolymer of sucrose and epichlorhydrin sold by Pharmacia Fine Chemicals, Piscataway, NJ) polyvinylpyrrolidone, and a denatured foreign DNA such as from calf thymus or salmon
20 sperm. The degree of complementarity between the sample and hybridization partner strands required for hybridization to occur depends on the stringency of the conditions. The extent and specificity of hybridization is affected by the following principal conditions.
25   1.   The purity of the nucleic acid preparation.
          2.   The content of G-C base pairs: G-C base pairs
                will exhibit greater thermal stability than A-T
                or A-U base pairs. Thus, hybrids with higher G-
                C content will be stable at higher temperatures.
30   3.   The length of the homologous base sequence: any
                short sequence of bases, e.g., less than 6
                bases, has a high degree of probability of
                recurring in many nucleic acids. Little or no
                specificity can be attained in hybridizations
35   involving such short sequences. The present

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hybridization partner sequence will preferably
be at least about 30 bases and up to about 100
bases for an oligonucleotide, or even greater
for a cDNA molecule.

5   4.   Ionic strength: the rate of reannealing
increases as the ionic strength of the incu-
bation solution increases. Thermal stability of
hybrids also increases.

5.   Incubation temperature: optimal reannealing
10   occurs at a temperature about 25-30°C below the
melting temperature (Tm) for a given duplex.
Incubation at temperatures significantly below
the optimum allows less related base sequences
to hybridize.

15   6.   Nucleic acid concentration and incubation time:
Normally, to drive the reaction towards
hybridization, one of the hybridizable sample
nucleic acid or hybridization partner nucleic
acid will be present in excess, usually 100-fold
20   excess or greater.

7.   Denaturing reagents: The presence of hydrogen
bond-disrupting agents such as formamide and
urea increases the stringency of hybridization.

8.   Incubation time: The longer the incubation
25   time, the more complete will be the hybrid-
ization.

9.   Volume exclusion agents: The presence of these
agents, as exemplified by dextran and dextran
sulfate, are through to increase the effective
30   concentrations of the hybridizing elements
thereby increasing the rate of resulting
hybridization.

Normally, the temperature conditions selected for
hybridization will be incompatible with the binding of the

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mismatch-binding protein to the formed hybrids or of antibodies or other binding partners to the mismatch-binding protein. Accordingly, the mismatch-binding protein and binding partner reagent binding steps and

5   label detection steps will proceed after completion of the hybridization step. The reaction mixture will usually be brought to a temperature in the range of from about 3°C to about 40°C and the mismatch-binding protein and additional reagent binding and detection steps then performed.

10   It may be expected in a particular assay using an RNA hybridization partner that the RNA will be subject to partial degradation by alkaline hydrolysis of the phosphodiester bonds or by the presence of ribonucleases (RNases). In the former case, hydrolysis can be

15 controlled by avoiding exposure of the hybridization partner to a pH higher than about 10. RNases can be effectively inhibited by the presence of such substances as sodium dodecylsulfate, aurintricarboxylic acid, ribonucleoside vanadyl complexes, heparin diethylpyro-

20 carbonate and proteinaceous RNase inhibitors isolated from mammalian sources.

        A mismatch-binding protein bound to the hybridization partner-target polynucleotide (DNA-DNA, DNA-RNA or RNA-RNA) hybrid can be detected either directly or indirectly

25 (see Figure 2). By direct detection is intended labeling the mismatch-binding protein with a detectable label, for example, radiolabels, fluorescent labels, conjugated enzymes, or the like, which are described in more detail below. Alternatively, as described above; the mismatch-

30 binding protein can be produced by recombinant DNA technology as a fusion protein with a fusion partner which can easily be detected. It is important that the fusion protein, as well as a detectably labeled or conjugated mismatch-binding protein, maintain the reactivity and

35 binding specificity of native, unmodified mismatch-binding

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protein. this reactivity and specificity can be ascer-
tained by one of ordinary skill in the art by routine
testing, for example, by the assay described herein.

By indirect detection is intended a method which
5   utilizes a binding partner specific for either the
mismatch-binding protein, for a reactive moiety conjugated
to the mismatch-binding protein, or for a fusion protein
partner of the mismatch-binding protein. When the
mismatch-binding protein is MutS, a preferred binding
10 partner is an anti-MutS antibody (or an antigen-binding
fragment thereof).

Any of the antibody reagents useful in the method of
the present invention may comprise whole antibodies,
antibody fragments, polyfunctional antibody aggregates, or
15 in general any substance comprising one or more specific
binding sites from an antibody. The antibody may be of
any immunoglobulin isotype, e.g., IgG, IgM, and so forth.
Any antigen-binding fragment of any such antibody can also
be employed, for instance, the Fab' or $F(ab')_2$ fragments.
20 In addition, aggregates, polymers, derivatives and
conjugates of immunoglobulins or their fragments can be
used where appropriate.

The immunoglobulin source for an antibody reagent can
be obtained in any manner such as by preparation of a
25 conventional polyclonal antiserum or by preparation of a
monoclonal or a chimeric antibody. Antiserum can be
obtained by well-established techniques involving
immunization of an animal, such as a mouse, rabbit, guinea
pig or goat, with an appropriate immunogen, e.g., the MutS
30 protein.

Alternatively, the binding partner may be an antibody
specific for the fusion protein partner, for example, β-
galactosidase, which is fused with a mismatch-binding
protein. In addition, an antibody specific for haptenic
35 group conjugated to the mismatch-binding protein molecule

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can be used. Another binding partner may be avidin or streptavidin, where the mismatch-binding protein has been modified by addition of biotin. In yet another embodiment, the binding partner may be a non-
5 immunoglobulin protein which has the property of binding to an immunoglobulin molecule, for example Staphylococcal protein A or Streptococcal protein G, which are well-known in the art. The binding partner may either itself be detectably labeled or may be detected indirectly by a
10 detectably labeled secondary binding partner, for example, a second antibody specific for the first antibody. Thus, if a rabbit-anti-mismatch-binding protein antibody serves as the first binding partner, a labeled goat-anti-rabbit immunoglobulin antibody would be a second binding partner.
15 In another embodiment, the signal generated by presence of the fusion protein partner of the mismatch-binding protein is amplified by reaction with a specific antibody for that fusion protein (e.g., an anti-β-galactosidase antibody) which is detectably labeled. One of ordinary skill in the
20 art can devise without undue experimentation a number of such possible first and second binding partner systems using conventional methods well-known in the art.

In yet another embodiment, the binding partner may be the MutL protein (Grilley, M. et al., J. Biol. Chem.
25 264:1000-1004 (1989); Modrich, P., 1989, supra; Lahue et al., 1989, supra) obtained from E. coli, S. typhimurium, or any other source, or a functional derivative thereof. A preferred functional derivative is a fusion protein between MutL and a second fusion partner protein which can
30 be readily detected, such as β-galactosidase. The purified MutL protein which has a molecular weight of 90 kDa is known to bind selectively to the complex between MutS and a nucleic acid heteroduplex in the presence of ATP. MutL does not bind directly to a mismatch-containing
35 heteroduplex nor to free MutS. The MutL protein has been

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cloned and expressed in bacteria (Pang et al., supra).
Thus, for use according to the present invention, the MutL
protein or functional derivative, preferably in detectably
labeled form, is added to the mutation detection assay in
5   the presence of ATP. The bound MutL is then detected.
Alternatively, unlabeled MutL may be used as the first
binding partner, followed by a labeled second binding
partner which binds to MutL, or by an unlabeled second
binding partner followed by a detectably labeled third
10   binding partner, as discussed above. As described above
for MutS binding partners, a second binding partner for
MutL may be an antibody specific for MutL, an antibody
specific for a hapten conjugated to MutL, avidin or
streptavidin where biotin is conjugated to MutL, an
15   antibody for the fusion partner protein of MutL, and the
like.
        Where the first or second binding partner described
above is an antibody, detection may be accomplished using
any of a variety of conventional immunoassays, including
20   enzyme immunoassays (EIA) (Voller, A., Diagnostic Horizons
2:1-7, 1978, Microbiological Associates Quarterly
Publication, Walkersville, MD; Voller, A. et al., J. Clin.
Pathol. 31:507-520 (1978); U.S. Reissue Pat. No. 31,006;
UK Patent 2,019,408; Butler, J.E., Meth. Enzymol. 73:482-
25   523 (1981); Maggio, E. (ed.), Enzyme Immunoassay, CRC
Press, Boca Raton, FL, 1980) or radioimmunoassays (RIA)
(Weintraub, B., Principles of Radioimmunoassays, Seventh
Training Course on Radioligand Assay Techniques, The
Endocrine Society, March 1986, pp. 1-5, 46-49 and 68-78).
30   In a preferred embodiment, a MutS-specific antibody
or antibody fragment is detectably labeled by linking the
same to an enzyme and use it in an EIA, or enzyme-linked
immunosorbent assay (ELISA). This enzyme, in turn, when
later exposed to a substrate in such a manner as to
35   produce a chemical moiety which can be detected, for

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example, by spectrophotometric, fluorometric or, most
preferably, by visual means. Preferably, the substrate is
a chromogenic substrate which generates a reaction product
visible to the naked eye.

5   Enzymes which can be used to detectably label the
binding partner for the mismatch-binding protein, such as
MutS-specific antibody, include, but are not limited to,
alkaline phosphatase, horseradish peroxidase, glucose-6-
phosphate dehydrogenase, staphylococcal nuclease, delta-V-
10 steroid isomerase, yeast alcohol dehydrogenase, alpha-
glycerophosphate dehydrogenase, triose phosphate
isomerase, asparaginase, ribonuclease, urease, catalase,
glucose-6-phosphate dehydrogenase, glucoamylase and
acetylcholinesterase.

15   By radioactively labeling the binding partner, for
example, the MutS-specific antibody, it is possible to
detect MutS bound to a solid support through the use of a
radioimmunoassay (RIA). The radioactive isotope can be
detected by such means as the use of a gamma counter or a
20 scintillation counter or by autoradiography. Isotopes
which are particularly useful for the purpose of the
present invention are: ^{3}H, ^{131}I, ^{35}S, ^{14}C, and preferably
^{125}I.

It is also possible to label the first or second
25 binding partner with a fluorescent compound. When the
fluorescently labeled antibody is exposed to light of the
proper wave length, its presence can then be detected due
to fluorescence. Among the most commonly used fluorescent
labelling compounds are fluorescein isothiocyanate,
30 rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-
phthaldehyde and fluorescamine.

The first or second binding partner also can be
detectably labeled by coupling it to a chemiluminescent
compound. The presence of the chemiluminescent-tagged
35 antibody is then determined by detecting the presence of

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luminescence that arises during the course of a chemical
reaction.   Examples of particularly useful
chemiluminescent labeling compounds are luminol, iso-
luminol, theromatic acridinium ester, imidazole, acri-
5   dinium salt and oxalate ester.

    Likewise, a bioluminescent compound may be used to
label the first or second binding partner. Biolumines-
cence is a type of chemiluminescence found in biological
systems in which a catalytic protein increases the
10   efficiency of the chemiluminescent reaction. The presence
of a bioluminescent protein is determined by detecting the
presence of luminescence. Important bioluminescent
compounds for purposes of labeling are luciferin,
luciferase and aequorin.

15   The test sample to be assayed can be in any medium of
interest, and will generally sample of medical,
veterinary, environmental, nutritional, or industrial
significance. Human and animal specimens and body fluids
particularly can be assayed by the present method,
20   providing that they contain cells from which nucleic acids
can be prepared. Preferred sources include blood, sperm,
other tissue, milk, urine, cerebrospinal fluid, sputum,
fecal matter, lung aspirates, throat swabs, genital swabs
and exudates, rectal swabs, and nasopharyngeal aspirates.
25   Where the sample contains principally double stranded
nucleic acids, such as the DNA naturally occurring in
cells, the sample will first be treated to release the
nucleic acid from the cells, which can be achieved by
mechanical disruption (such as freeze/thaw, abrasion,
30   sonication), physical/chemical disruption, such as
treatment with detergents (e.g., Triton, Tween, or sodium
dodecylsulfate), osmotic shock, heat, or enzymatic lysis
(lysozyme, proteinase K, pepsin, etc.)

    According to the method of the present invention, the
35   release dsDNA may be subjected to cleavage by restriction

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endonuclease enzymes to form fragments of the appropriate
length for the assay, and which contain the desired
sequence. The choice of restriction enzymes depends on
the desired target sequence and the presence of
5   appropriate restriction enzyme recognition sites on either
side of that sequence. One of ordinary skill in the art
will be able to identify the site and select the enzymes
without undue experimentation. See, for example,
Sambrook, J. et al., supra.
10   Following restriction enzyme digestion, denaturation
of nucleic acids is preferably accomplished by heating in
boiling water or alkali treatment (e.g., 0.1 N sodium
hydroxide). The resulting test medium will contain
nucleic acids in single stranded form which can then be
15   assayed according to the method of the present invention.
          The present invention also provides methods and kits
for detecting particular mutations in humans. Table 1,
below, provides a non-limiting list of human mutations
which can be detected using the methods of the present
20   invention. See, also, Cooper, D.N. et al., Hum. Genet.
85:55-74 (1990), which is hereby incorporated by reference
in its entirety.

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TABLE 1

POINT MUTATIONS CAUSING HUMAN GENETIC DISEASES

DISEASE	MUTATION				REFERENCE				
	Gene	Base change	Amino ac. change	CD	Author	Journal	Vol.	Page	Yr.
ADA Deficiency	ADA	CGG-CAG	Arg-Gln	101	Bonthron	JCI	76	894	85
ADA Deficiency	ADA	AAA-AGA	Lys-Arg	80	Valerio	EmboJ	5	113	86
ADA Deficiency	ADA	CTG-CGG	Leu-Arg	304	Valerio	EmboJ	5	113	86
ADA Deficiency	ADA	gCGG-TGG	Arg-Trp	101	Akeson	JBC	263	16291	88
ADA Deficiency	ADA	CGT-CAT	Arg-His	211	Akeson	JBC	263	16291	88
ADA Deficiency	ADA	GCG-GTG	Ala-Val	329	Akeson	PNAS	84	5947	87
ADA Deficiency	ADA	CCG-CAG	Pro-Gln	297	Hirshhorn	JCI	83	497	89
Adenylate kinase def.	AK	cCGG-TGG	Arg-Trp	128	Matsuura	JBC	264	10148	89
Adrenal hyperplasia	CA21HB	CCC-CGC	Pro-Arg	426	Matteson	PNAS	84	5858	87
Adrenal hyperplasia	CA21HB	AGC-ACC	Ser-Thr	269	Rodrigues	EmboJ	6	1653	87
Adrenal hyperplasia	CA21HB	ATC-AAC	Ile-Asn	172	Amor	PNAS	85	1600	88
Adrenal hyperplasia	CA21HB	gCAG-TAG	Gln-Term	318	Globerman	JCI	82	139	88
Adrenal hyperplasia	CA21HB	cGTG-CTG	Val-Leu	211	Speiser	NEJM	319	19	88
Adrenal hyperplasia	CA21HB	cGTG-TTG	Val-Leu	281	Speiser	NEJM	319	19	88
Adrenal hyperplasia	CA21HB	AAC-AGC	Asp-Ser	494	Rodrigues	EmboJ	6	1653	87
Aldolase A def.	ALDA	GAT-GGT	Asp-Gly	128	Kishi	PNAS	84	8623	88
Aldolase B def.	ALDB	tGCT-CCT	Ala-Pro	149	Cross	Cell	53	881	88
Amyloidotic poly.	PALB	cGTG-ATG	Val-Met	30	Furuya	JCI	80	1706	87
Amyloidotic poly.	PALB	TCT-TAT	Ser-Tyr	77	Wallace	JCI	81	189	88
Amyloidotic poly.	PALB	ATC-AGC	Ile-Ser	84	Wallace	AJIIG	43	182	88
Angioneurotic edema	CII	gCGC-TGC	Arg-Cys	444	Skriver	JBC	264	3066	89
Angioneurotic edema	CII	CGC-CAC	Arg-His	444	Skriver	JBC	264	3066	89
Antithrombin III Def.	AT3	CGT-TGT	Arg-Cys	47	Duchange	NAR	14	2408	86
Antithrombin III Def.	AT3	CCG-CTG	Pro-Leu	41	Chang	JBC	261	1174	86
Antithrombin III Def.	AT3	CCT-CTT	Pro-Leu	407	Bock	Biochem	27	6171	88
Antithrombin III Def.	AT3	aGCA-ACA	Ala-Thr	382	Devraj-K.	Blood	72	1518	88
Antithrombin III Def.	AT3	cCGT-TGT	Arg-Cys	393	Thein	Blood	72	1817	88
Antithrombin III Def.	AT3	CGT-CAT	Arg-His	393	Thein	Blood	72	1817	88
Antithrombin III Def.	AT3	CGT-CCT	Arg-Pro	393	Lane	JBC	264	10200	89

Table 1 (continued)

DISEASE	MUTATION				REFERENCE				
	Gene	Base change	Amino ac. change	CD	Author	Journal	Vol.	Page	Yr.
α1-Antitrypsin def.	PI	GTG-GCG	Val-Ala	213	Nukiwa	JBC	261	15989	86
α1-Antitrypsin def.	PI	cGAG-AAG	Glu-Lys	342	Kidd	Nature	304	230	83
α1-Antitrypsin def.	PI	cCCT-TCT	Pro-Leu	369	Hofker	Hum Genet	81	264	89
α1-Antitrypsin def.	PI	gAAG-TAC	Lys-Term	217	Satoh	AJHG	42	77	88
α1-Antitrypsin def.	PI	CTG-CCG	Leu-Pro	41	Takahashi	JBC	263	15528	88
APRT deficiency	APRT	ATG-ACG	Met-Thr	136	Hidaka	JCI	81	945	88
ApoA1 deficiency	APOA1	GAGC-GAT	Glu-Asp	120	Law	JBC	260	12810	85
ApoB deficiency	APOB	aCAA-TAA	Gln-Term	2153	Hospatta	BBRC	148	279	87
ApoB deficiency	APOB	tCGA-TGA	Arg-Term	1306	Collins	NAR	16	8361	88
ApoB deficiency	APOB	CGG-CAG	Arg-Gln	3500	Soria	PNAS	86	587	89
ApoB deficiency	APOB	tCGA-TGA	Arg-Term	2058	Young	NEJM	320	1604	89
ApoE deficiency	APOE	gCGC-TGC	Arg-Cys	158	Funke	Clin Chem	32	1285	86
ApoE deficiency	APOE	gCGC-AGC	Arg-Ser	136	Eml	Genomics	3	373	88
ApoE deficiency	APOE	gCGT-TGT	Arg-Cys	145	Eml	Genomics	3	373	88
ApoE deficiency	APOE	gGAG-AAG	Glu-Lys	244	Tajima	J Biochem	105	249	89
ApoE deficiency	APOE	gCGC-TGC	Arg-Cys	142	Rall	JCI	83	1095	89
Diabetes (ins. res.)	INSR	AGGt-AGT	Arg-Ser	735	Yoshimasa	Science	240	784	88
Diabetes (ins. res.)	INSR	TGG-TCG	Trp-Ser	1200	Moller	NEJM	319	1526	88
Diabetes (MODY)	INS	TTC-TCC	Phe-Ser	24	Haneda	PNAS	80	6366	83
Diabetes (NIDDM)	INSR	GGC-GTC	Gly-Val	996	Odawara	Science	245	66	89
Ehlers-Danlos IV	COLIA3	gGGT-AGT	Gly-Ser	790	Tromp	JBC	264	1349	89
Ehlers-Danlos VII	COLIA1	ATGg-ATA	Met-Ile	159	Weil	EmboJ	8	1705	89
Elliptocytosis	SPTA1	AGTg-AGG	Ser-Arg	39	Garbarz	Blood	72S	41A	88
Fabry disease	GLA	cCGG-TGG	Arg-Trp	356	Bernstein	JCI	83	1390	89
Factor X deficiency	FX	cCGC-TGC	Arg-Cys	366	Jagadees	JCBS	13E	291	89
Factor XI deficiency	FXI	aGAA-TAA	Glu-Term	117	Asakai	PNAS	86	7667	89
Gangliosidosis GM2	HEXB	CGC-CAC	Arg-His	178	Ohno	AJHG	41	A231	87
Gangliosidosis GM2	HEXB	aGGT-AGT	Gly-Ser	269	Paw	PNAS	86	2413	89
G6PD deficiency	G6PD	gAAT-GAT	Asn-Asp	126	Vulliamy	PNAS	85	5171	88
G6PD deficiency	G6PD	gGAT-AAT	Asp-Asn	58	Vulliamy	PNAS	85	5171	88
G6PD deficiency	G6PD	cGAG-AAG	Glu-Lys	156	Vulliamy	PNAS	85	5171	88
G6PD deficiency	G6PD	cGCC-ACC	Ala-Thr	335	Vulliamy	PNAS	85	5171	88
G6PD deficiency	G6PD	TCC-TTC	Ser-Phe	188	Vulliamy	PNAS	85	5171	88

Table 1 (continued)

DISEASE	MUTATION				REFERENCE				
	Gene	Base change	Amino ac. change	CD	Author	Journal	Vol.	Page	Yr.
G6PD deficiency	G6PD	CGGG-AGG	Gly-Arg	447	Vulliamy	PNAS	85	5171	88
G6PD deficiency	G6PD	CGTG-ATG	Val-Met	68	Vulliamy	PNAS	85	5171	88
G6PD deficiency	G6PD	aGAT-CAT	Asp-His	282	De Vita	AJHG	44	233	89
G6PD deficiency	G6PD	aGGC-AGC	Gly-Ser	163	Vulliamy	NAR	17	5868	89
Gaucher's (type 1)	GBA	CGG-CAG	Arg-Gln	119	Graves	DNA	7	521	88
Gaucher's (type 1)	GBA	AAC-AGC	Asn-Ser	370	Tsuji	PNAS	85	2349	88
Gaucher's (type 2)	GBA	CTG-CCG	Leu-Pro	444	Tsuji	NEJM	316	570	87
Gaucher's (type 2)	GBA	CCC-CGC	Pro-Arg	415	Wldsgerson	AJHG	44	365	89
Gerstmann-Straeuss	PRP	CCG-CTG	Pro-Leu	102	Hsiao	Nature	338	342	89
Gyrate Atrophy	OAT	ATGt-ATA	Met-Ile	1	Mitchell	JCI	81	630	88
Gyrate Atrophy	OAT	AACt-AAA	Asn-Lys	54	Ramesh	PNAS	85	3777	88
Gyrate Atrophy	OAT	aGTG-ATG	Val-Met	332	Ramesh	PNAS	85	3777	88
Gyrate Atrophy	OAT	AGG-ACG	Arg-Thr	180	Mitchell	PNAS	86	197	89
Gyrate Atrophy	OAT	CTT-CCT	Leu-Pro	402	Mitchell	PNAS	86	197	89
Heparin cofactor2 Def.	HCF2	CGC-CAC	Arg-His	189	Blinder	JBC	264	5128	89
HPRT deficiency	HPRT	tCGA-GGA	Arg-Gly	50	Wilson	JCI	72	767	83
HPRT deficiency	HPRT	ATTg-ATG	Ile-Met	131	Fujimori	Hum Genet	79	39	88
HPRT deficiency	HPRT	GTC-GAC	Val-Asp	129	Davidscn	Gene	68	85	88
HPRT deficiency	HPRT	AGCt-AGA	Ser-Arg	103	Cariello	AJHG	42	726	88
HPRT deficiency	HPRT	TCA-TTA	Ser-Leu	109	Davidson	JCI	82	2164	88
HPRT deficiency	HPRT	GAT-GGT	Asp-Gly	200	Davidson	JBC	264	520	89
HPRT deficiency	HPRT	TTCt-TTA	Phe-Leu	73	Gibbs	PNAS	86	1919	89
HPRT deficiency	HPRT	CGCA-TCA	Ala-Ser	160	Gibbs	PNAS	86	1919	89
HPRT deficiency	HPRT	TTG-TCG	Leu-Ser	130	Gibbs	PNAS	86	1919	89
HPRT deficiency	HPRT	aCGA-TGA	Arg-Term	169	Gibbs	PNAS	86	1919	89
HPRT deficiency	HPRT	CTTC-GTC	Phe-Val	198	Gibbs	PNAS	86	1919	89
HPRT deficiency	HPRT	TGT-TAT	Cys-Tyr	205	Gibbs	PNAS	86	1919	89
HPRT deficiency	HPRT	tCAT-GAT	His-Asp	203	Gibbs	PNAS	86	1919	89
HPRT deficiency	HPRT	gGGC-CGC	Gly-Arg	70	Fujimori	JCI	83	11	89
HPRT deficiency	HPRT	CTA-CCA	Leu-Pro	40	Davidson	JCI	84	342	89
HPRT deficiency	HPRT	GGG-GAG	Gly-Glu	69	Davidson	JCI	84	342	89
HPRT deficiency	HPRT	GAT-GTT	Asp-Val	79	Davidson	JCI	84	342	89
HPRT deficiency	HPRT	CGCA-TCA	Ala-Ser	160	Davidson	JCI	84	342	89

Table 1 (continued)

DISEASE	MUTATION				REFERENCE				
	Gene	Base change	Amino ac. change	CD	Author	Journal	Vol.	Page	Yr.
HPRT deficiency	HPRT	cTTC-GTC	Phe-Val	198	Davidson	JCI	84	342	89
Hypophosphatasia	ALPL	cGCC-ACC	Ala-Thr	162	Weiss	PNAS	85	7666	88
Hypercholesterolemia	LDRL	CCG-CTG	Pro-Leu	664	Soutar	PNAS	86	4166	89
Hyperproinsulinemia	INS	aCAC-GAC	His-Asp	B10	Chan	PNAS	84	2194	87
Hyperproinsulinemia	INS	TTct-TTG	Phe-Leu	24	Kwok	BBRC	98	844	81
Hyperproinsulinemia	INS	TTct-TTG	Phe-Leu	B25	Kwok	Diabetes	32	872	83
Hyperproinsulinemia	INS	CGT-CAT	Arg-His	65	Shibasaki	JCI	76	378	85
Hyperproinsulinemia	INS	tGTG-TTG	Val-Leu	A3	Awata	Diabetes	37	1068	88
Immunoglobulin K def.	IGK	cTGC-GGC	Cys-Gly	194	Stavnezer	Science	230	458	85
Immunoglobulin K def.	IGK	gTGG-CGG	Trp-Arg	148	Stavnezer	Science	230	450	85
LDLR deficiency	LDLR	TGGc-TGA	Trp-Term	792	Lehrman	Cell	41	735	85
LDLR deficiency	LDLR	TAT-TGT	Tyr-Cys	807	Davis	Cell	45	15	86
LDLR deficiency	LDLR	TGCc-TGA	Cys-Term	660	Lehrman	JBC	262	401	87
Leprechaunism	INSR	gAAG-GAG	Lys-Glu	460	Kadowaki	Science	240	787	88
Leprechaunism	INSR	cCAG-TAG	Gln-Term	672	Kadowaki	Science	240	787	88
Maple syrup urine dis.	BCKD	cTAC-AAC	Tyr-Asn	394	Zhang	JCI	83	1425	89
Osteogen. imperf. (1)	COLIA1	tGGT-TGT	Gly-Cys	748	Vogel	JBC	262	14737	87
Osteogen. imperf. (1)	COLIA1	tGGT-TGT	Gly-Cys	1017	Labhard	MBM	5	197	88
Osteogen. imperf. (2)	COLIA2	GGT-GAT	Gly-Asp	907	Baldwin	JBC	264	3002	89
Osteogen. imperf. (2)	COLIA1	cGGA-AGA	Gly-Arg	664	Bateman	JBC	263	11627	88
Osteogen. imperf. (2)	COLIA2	GGT-GTT	Gly-Val	256	Patterson	JBC	264	10081	89
Osteogen. imperf. (4)	COLIA1	tGGT-AGT	Gly-Ser	832	Marini	JBC	264	11893	89
Osteogen. imperf. (2)	COLIA1	tGGC-TGC	Gly-Cys	904	Constanti	JCI	83	574	88
OTC deficiency	OTC	CGA-CAA	Arg-Gln	109	Maddelena	JCI	82	1353	88
OTC deficiency	OTC	CGA-CAA	Arg-Gln	26	Grompe	JBCS	13D	41	89
OTC deficiency	OTC	gCAG-GAG	Arg-Glu	216	Grompe	PNAS	86	5888	89
OTC deficiency	OTC	aGAA-TAA	Glu-Term	154	Grompe	PNAS	86	5888	89
Phenylketonuria	PAH	tCGG-TGG	Arg-Trp	408	Dilella	Nature	327	333	07
Phenylketonuria	PAH	CTG-CCG	Leu-Pro	311	Licht.-K.	Biochem	27	2881	88
Phenylketonuria	PAH	cGAA-AAA	Glu-Lys	280	Lynnet	AJHG	44	511	89
Porphyria	UROD	GGG-GAG	Gly-Glu	281	De Verne	Science	234	732	86
Porphyria	UROD	GGG-GTG	Gly-Val	281	Garey	Blood	73	892	89

Table 1 (continued)

DISEASE	Gene	MUTATION			REFERENCE				
		Base change	Amino ac. change	CD	Author	Journal	Vol.	Page	Yr.
Protein C deficiency	PROC	cCGA-TGA	Arg-Term	306	Romeo	PNAS	84	2829	87
Protein C deficiency	PROC	TGGa-TGC	Trp-Cys	402	Romeo	PNAS	84	2829	87
Protein C deficiency	PROC	gCGG-TGG	Arg-Trp	169	Matsuda	NEJM	319	1265	88
PNP deficiency	NP	tGAA-AAA	Glu-Lys	89	Williams	JBC	262	2332	87
Rickets(vit.D resist)	VDR	CGA-CAA	Arg-Gln	?	Hughes	Science	242	1702	89
Tay-Sachs disease	HEXB	CGC-CAC	Arg-His	178	Ohno	J Nchem	50	316	88
Tay-Sachs disease	HEXB	cGAA-AAA	Glu-Lys	482	Nakano	J Nchem	51	984	88
Tay-Sachs disease	HEXB	aGGT-AGT	Gly-Ser	269	Navon	Science	243	1471	89
TPI deficiency	TPI	GAGt-GAC	Glu-Asp	104	Daar	PNAS	83	7903	86
TSH deficiency	TSHB	tGGA-AGA	Gly-Arg	29	Hayashiz.	EmboJ	8	2291	89
von Willebrand 2a	VWF	GTC-GAC	Val-Asp	844	Ginsburg	PNAS	86	3723	89
von Willebrand 2a	VWF	cCGG-TGG	Arg-Trp	834	Ginsburg	PNAS	86	3723	89

Abbreviations:

ADA- adenosine deaminase; AJHG- Am. J. Hum. Genet.; Apo- apolipoprotein; APRT-
adenosinephosphoribosyltrasferase; BBRC- Biochem. Biophys. Res. Comm.; CD- codon;
G6PD- glucose-6-phosphate dehdrogenase; HPRT- hypoxanthinephosphoribosyltransferase;
JBC- J. Biol. Chem.; JCI- J. Clin. Invest.; JCBS- J. Cell.Biol. (Suppl); J Nchem- J.
Neurochem.; LDLR- low density lipoprotein receptor; MBM- Mol. Biol. Med.; MODY-
maturity-onset diabetes of the young; NAR- Nucl. Acids. Res.; NEJM- New Eng. J. Med.;
NIDDM- non-insulin-dependent diabetes mellitus; OTC- ornithine transcarbamylase; PNAS-
Proc. Natl. Acad. Sci. USA; PNP- purine nucleoside phosphorylase; TPI- triose phosphate
isomerase; TSH- thyroid stimulating hormone

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.The present invention is also directed to a kit or
reagent system useful for practicing the method described
above. Such a kit will contain a reagent combination
comprising the essential elements required to conduct an
5   assay according to the methods disclosed herein. The
reagent system is presented in a commercially packaged
form, as a composition or admixture where the
compatibility of the reagents will allow, in a test device
configuration, or more typically as a test kit, i.e., a
10 packaged combination of one or more containers, devices,
or the like holding the necessary reagents, and usually
including written instructions for the performance of
assays. The kit of the present invention may include any
configurations and compositions for performing the various
15 assay formats described herein.

In all cases, the reagent system will comprise (1) an
immobilizable or immobilized hybridization partner as
described herein, and (2) a mismatch-binding protein or
functional derivative. The mismatch-binding protein may
20 optionally be detectably labeled. The kit may optionally
contain a first binding partner for the mismatch-binding
protein, e.g., an anti-MutS antibody, a MutL protein or
functional derivative, and any additional binding partners
or detectable label reagents. A kit according to the
25 present invention can additionally include ancillary
chemicals such as the components of the hybridization
solution, a denaturation agent capable of converting
double stranded nucleic acids in a test sample into single
stranded form, or restriction enzymes for producing
30 fragments of the sample nucleic acid.

Having now generally described the invention, the
same will be more readily understood through reference to
the following examples which are provided by way of
illustration, and are not intended to be limiting of the
35 present invention.

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EXAMPLE 1
ASSAY OF A SAMPLE FROM BLOOD FOR A MUTANT DNA SEQUENCE

The assay format is described generally in Figure 3.
Sample DNA is obtained from blood cells being tested for
5 the presence of a particular mutation, which differs from
wild-type by a single-base pair or by an addition or
deletion of one or a few base pairs. The DNA is then
denatured at which point it is ready to be assayed.
A test DNA hybridization partner of about 30
10 nucleotides is constructed to have a sequence corre-
sponding to the wild-type gene sequence around the
mutation of interest. As a positive control for a
mismatch, the point mutation of the sickle cell hemoglobin
is utilized. An oligonucleotide having the mutant
15 sequence (exon 10) is produced. As a negative control for
the assay, an oligonucleotide known to be of the wild-type
sequence, the wild-type human hemoglobin sequence of exon
10 is produced.
The hybridization partner DNA is immobilized on
20 nitrocellulose filters as follows:
(a) Test hybridization partner DNA in the form of a
vertical line of a "plus" sign.
(b) Positive control DNA is immobilized on the filter in
the form of a horizontal member of the same "plus" sign.
25 (c) Negative control DNA is applied to the filter in the
form of a dot near the "plus" sign.
The filters are treated to fix the oligonucleotides
and to block all unreacted sites to prevent non-specific
binding of DNA and proteins during the assay, using
30 conventional methods (Sambrook et al., supra).
The sample DNA is added in a concentration between 10
and 100 µg/ml and the hybridization reaction is allowed to
proceed at 45-55°C in 0.1-0.3 M NaCl, resulting in the

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appropriate restriction fragments binding to the three
oligonucleotide fragments.

The filter is washed and MutS is added to the filter
and allowed to bind, and the filter is again washed.

5   Polyclonal rabbit anti-MutS antibodies are added to
the filter, allowed to bind, and unbound antibodies washed
off.

A horseradish peroxidase-conjugated goat anti-rabbit
immunoglobulin antibody is then added, allowed to bind,
10 and unbound antibody washed off.

A chromogenic substrate for horseradish peroxidase is
added and the color reaction allowed to develop until a
colored line (or plus sign) appears, at which time the
reaction is stopped.

15   The results will appear as follows:
A.   For a sample lacking the point mutation in the DNA (a
negative):
(1)   The dot (negative control hybridization partner) is
not visible, indicating that the assay was not a false
20 positive.
(2)   Only the horizontal line is visible. Since the
subject presumably has a normal Hb gene, and the positive
control oligonucleotide hybridization partner had the
mutant sequence, a reaction has occurred.
25 (3)   Thus a colored "minus" sign appears on the filter,
indicating a negative result in the test for mutant DNA in
the sample.
B.   For a sample having the point mutation in the DNA (a
positive):
30 (1)   The dot (negative control hybridization partner) is
not visible, indicating that the assay was not a false
positive.
(2)   A "plus sign" is visible. The horizontal line is
visible, since the subject presumably has a normal Hb
35 gene, and the positive control oligonucleotide hybrid-

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ization partner had the mutant sequence, resulting in a reaction. Because the DNA from this sample has a point mutation in the target DNA sequence, the vertical line of the "plus sign" yields a colored reaction product.

5   EXAMPLE II
                ASSAY FOR A MUTANT DNA SEQUENCE
                USING A MutL BINDING PARTNER

        The general assay format is similar to that described
     in Example I. Sample DNA is obtained from blood cells
10   being tested for the presence of a particular mutation,
     which differs from wild-type by a single-base pair or by
     an addition or deletion of one or a few base pairs. The
     DNA is then denatured at which point it is ready to be
     assayed.
15   A test DNA hybridization partner of about 30
     nucleotides is constructed to have a sequence corre-
     sponding to the wild-type gene sequence around the
     mutation of interest. As a positive control for a
     mismatch, the point mutation of the sickle cell hemoglobin
20   mutation is utilized. An oligonucleotide having the
     mutant sequence (exon 10) is produced. As a negative
     control for the assay, an oligonucleotide known to be of
     the wild-type sequence, the wild-type human hemoglobin
     sequence of exon 10 is produced.
25   The hybridization partner DNA is immobilized on
     nitrocellulose filters as follows:
     (a) Test hybridization partner DNA in the form of a
     vertical line of a "plus" sign.
     (b) Positive control DNA is immobilized on the filter in
30   the form of a horizontal member of the same "plus" sign.
     (c) Negative control DNA is applied to the filter in the
     form of a dot near the "plus" sign.

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The filters are treated to fix the oligonucleotides and to block all unreacted sites to prevent non-specific binding of DNA and proteins during the assay, using conventional methods (Sambrook et al., supra).

5   The sample DNA is added in a concentration between 10 and 100 µg/ml and the hybridization reaction is allowed to proceed at 45-55°C in 0.1-0.3 M NaCl, resulting in the appropriate restriction fragments binding to the three oligonucleotide fragments.

10   The filter is washed and MutS is added to the filter and allowed to bind, and the filter is again washed.

A MutL-β-galactosidase fusion protein is added as a first binding partner and allowed to bind, and unbound material washed off.

15   The second binding partner, a polyclonal rabbit anti-β-galactosidase antiserum is added to the filter, allowed to bind, and unbound antibodies washed off.

The third binding partner, a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin antibody is 20 then added, allowed to bind, and unbound antibody washed off.

A chromogenic substrate for horseradish peroxidase is added and the color reaction allowed to develop until a colored line (or plus sign) appears, at which time the 25 reaction is stopped.

The results will appear as follows:
A. For a sample lacking the point mutation in the DNA (a negative):
(1) the dot (negative control hybridization partner) is 30 not visible, indicating that the assay was not a false positive.
(2) Only the horizontal line is visible. Since the subject presumably has a normal Hb gene, and the positive control oligonucleotide hybridization partner had the 35 mutant sequence, a reaction has occurred.

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(3) Thus a colored "minus" sign appears on the filter, indicating a negative result in the test for mutant DNA in the sample.

B. For a sample having the point mutation in the DNA (a
5 positive):

(1) the dot (negative control hybridization partner) is not visible, indicating that the assay was not a false positive.

(2) A "plus" sign is visible. Because the DNA from this
10 sample has a point mutation in the target DNA sequence, the vertical line of the "plus sign" yields a colored reaction product.

EXAMPLE III
DETECTION OF A MUTATED p53 ONCOGENE IN A TISSUE SAMPLE

15   The p53 gene appears to be a tumor suppressor gene (Levine, A. et al., Nature 351:453-455 (1991)) wherein mutation at numerous sites can result in expression of a tumor, in particular colorectal carcinoma. See, also, for example, Kinzler, K. W. et al., Science 251:1366-1370
20 (1991); Kern, S. E. et al., Oncogene 6:131-136 (1991); Vogelstein, B., Nature 348:681-682 (1990); Baker, S. J. et al., Science 249:912-915 (1990).

Human DNA is isolated from tumor tissue. The isolated DNA is sheared under standard conditions. The
25 DNA is then denatured at which point it is ready to be assayed.

A cDNA molecule corresponding to the p53 gene is produced by standard methods (Sambrook et al., supra) and serves as the hybridization partner. Regions harboring
30 known mutations may be scattered throughout the p53 sequence.

The hybridization partner DNA is immobilized on nitrocellulose filters as follows (see Figure 3):

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(a) .The test hybridization partner (p53 cDNA) is spotted in the form of a vertical line of a "plus" sign.

(b) Positive control DNA, is immobilized on the filter in the form of a horizontal member of the same "plus" sign.

5 (c) Negative control DNA is applied to the filter in the form of a dot near the "plus" sign.

The filters are treated to fix the hybridization partner poly- or oligonucleotides and to block all unreacted sites to prevent non-specific binding of DNA and 10 proteins during the assay.

The sample DNA is added and the reaction is incubated under hybridizing conditions (as described above), except that the hybridization temperature is 55-60°C, resulting in the appropriate DNA fragments binding to the three 15 hybridization partner poly- or oligonucleotides.

The filter is washed and MutS is added to the filter and allowed to bind, and the filter is again washed.

Polyclonal rabbit anti-MutS antibodies are added to the filter, allowed to bind, and unbound antibody washed 20 off.

A horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin antibody is then added, allowed to bind, and unbound antibody washed off.

A chromogenic substrate for horseradish peroxidase is 25 added and the color reaction allowed to develop until a colored "minus" or "plus" sign appears, at which time the reaction is stopped.

The results will appear as follows:

A. For a sample lacking the mutant p53 oncogene:

30 (1) The dot (negative control hybridization partner) is not visible, indicating that the assay was not a false positive.

(2) Only the horizontal line is visible. Since the subject presumably has a non-mutated sequence comple-35 mentary to the positive control DNA, and the positive

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control oligonucleotide hybridization partner has a <u>mutant</u> sequence, a reaction occurs.

(3) Thus a colored "minus" sign appears on the filter, indicating a negative result in the test for a mutant p53
5 oncogene in the sample DNA.

B. For a sample having a mutant p53 oncogene:

(1) The dot (negative control hybridization partner) is not visible, indicating that the assay was not a false positive.

10 (2) A "plus" sign is visible. The horizontal line is visible, since the subject presumably has a normal Hb gene, and the positive control oligonucleotide hybrid- ization partner had the <u>mutant</u> sequence, resulting in a reaction. Because the DNA from this sample has at least
15 one point mutation in the target DNA sequence, the vertical line of the "plus sign" yields a colored reaction product.

<u>EXAMPLE IV</u>

<u>DETECTION OF EXPRESSION OF A MUTATED p53 ONCOGENE</u>

20   Human mRNA is isolated from tumor tissue by con- ventional methods (see, Sambrook <u>et al.</u>, <u>supra</u>). A cDNA molecule corresponding to the p53 gene is produced by standard methods (Sambrook <u>et al.</u>, <u>supra</u>) and serves as the hybridization partner. The assay format is described
25 generally in Figure 3.

The hybridization partner DNA is immobilized on nitrocellulose filters as follows:

(a) The test hybridization partner (p53 DNA) is spotted in the form of a vertical line of a "plus" sign.

30 (b) Positive control DNA comprises an oligonucleotide sequence corresponding to a gene known to be expressed in the tumor tissue, which has had one base modified. This DNA is immobilized on the filter in the form of a horizontal member of the same "plus" sign.

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(c) ·Negative control DNA, comprising the wild-type sequence of the oligonucleotide used as the positive control, is applied to the filter in the form of a dot near the "plus" sign.

5   The filters are treated to fix the hybridization partner poly- or oligonucleotides and to block all unreacted sites to prevent non-specific binding of nucleic acid and proteins during the assay.

The sample mRNA is added and the reaction is
10 incubated under hybridizing conditions, resulting in mRNA binding to the three hybridization partner poly- or oligonucleotides.

The filter is washed and MutS is added to the filter and allowed to bind, and the filter is again washed.
15   Polyclonal rabbit anti-MutS antibodies are added to the filter, allowed to bind, and unbound antibody washed off.

A horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin antibody is then added, allowed to bind,
20 and unbound antibody washed of.

A chromogenic substrate for horseradish peroxidase is added and the color reaction allowed to develop until a colored "minus" or "plus" sign appears, at which time the reaction is stopped.
25   The results will appear as follows:

A. For a sample expressing the wild-type p53 gene:

(1) The dot (negative control hybridization partner) is not visible, indicating that the assay was not a false positive.
30 (2) Only the horizontal line is visible. Thus a colored "minus" sign appears on the filter, indicating a negative result in the test for expression of a mutant p53 oncogene in the sample mRNA.

B. For a sample expressing a mutant p53 gene:

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(1) The dot (negative control hybridization partner) is
not visible, indicating that the assay was not a false
positive.

(2) A "plus sign" is visible. The horizontal line is
5 visible, since the subject presumable has a non-mutated
sequence complementary to the positive control DNA, and
the positive control oligonucleotide hybridization partner
has a _mutant_ sequence, a reaction occurs. Because the
mRNA from this sample has at least one point mutation in
10 the target sequence, the vertical line of the "plus sign"
yields a colored reaction product.

EXAMPLE V

MISMATCH DETECTION BY mutS PROTEIN
MATERIALS AND METHODS
15   _Cells_. 1.2L (lambda cI857 N^t ΔH kil$^-$) was made a
mutS overproducer strain by the introduction of the mutS
expressing plasmid pMS312 (Su and Modrich, Proc. Nat.
Acad. Sci. USA. $\underline{83}$:5057, 1986).

     Purification of mutS. The mutS purification scheme
20 is a modification of that described by Su and Modrich
(ibid.). 1.2L (pMS312) cells were grown at 30°C in 8
liters of 869 medium (10 g/l Bacto-Tryptone (Difco), 5 g/l
yeast extract (Difco), 5 g/l NaCl) containing 50 µg/ml
ampicillin to an OD_{590} of 1.3-1.8. Cultures were diluted
25 1:2 in 869 medium prewarmed to 60°C and incubated at 42°C
for 5 hours. Cultures were chilled, the cells harvested
by centrifugation and the cell past stored at -70°C. Cell
past (15-29 gm) was thawed on ice and the cells
resuspended in an equal volume of buffer A (20mM KPO_4,
30 pH7.4, 1mM EDTA, 1mM PMSF, 10 mM 2-mercaptoethanol).
Cells were lysed by sonication in an ice-acetone bath.
The lysate was clarified by centrifugation (40,000g; 30
minutes). The supernatant was treated with 1/4 volume 25%

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(w/v) streptomycin sulfate in buffered A and stirred for
45 minutes at 4°C. Insoluble material was removed by cen-
trifugation (40,000g; 30 minutes). Ammonium sulfate (0.2g
per ml of supernatant) was added over a 15 minute period
5 and the suspension stirred for an additional 40 minutes at
4°C. The precipitate was collected by centrifugation
(40,000g; 30 minutes) and resuspended in 10ml of buffer A.
1ml fractions were diluted 1:10 in buffer B (20mM KPO$_4$, pH
7.4, 0.1mM EDTA, 1mM PMSF, 10mM 2-mercaptoethanol)
10 containing 0.025M kCL and applied immediately at 2 ml/min
to a 1.5 x 30cm Heparin-Separose CL6B (Pharmacia) column.
Column was washed with 100ml buffer B containing 0.1M KCl
and eluted with a 300 ml linear gradient of KCl (0.1-0.5M)
in buffer B. 100 3ml fractions were collected. Samples
15 were run on 4-20% SDS-PAGE gels under reducing conditions
and those fractions containing >95% mutS protein (97 kDa)
were pooled. Glycerol was added to the pooled material to
a final concentration of 20% and aliquots were stored at -
70°C.

20   Oligonucleotides. Oligonucleotides were obtained
from Biopolymer Labs. The sequence of the oligonucleo-
tides was taken from the 30 base region surrounding the
site of sickle cell mutation in the human β-globin gene.
In order to allow oligonucleotides to be immobilized on
25 nitrocellulose filters, selected oligonucleotides were
prepared with a 5' 25mer poly-C "tail" and a 5' amino-
modifier. The sequences used in these experiments are
presented in Table 2. (Note: The actual sickle cell
mutation is an A:T -> T:A transversion.) Oligonucleotides
30 were annealed in various combinations to produce G:T, A:C
and G:G mismatches, a heteroduplex with one unpaired base
(such as would be formed by annealing an oligonucleotide
with a wild type sequence to one with a one base addition
or deletion mutation) and two different homoduplexes,

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i.e., perfectly complementary duplexes. Annealing
conditions are described below.

    Anti-mutS antibodies. Polyclonal anti-mutS
antibodies were prepared from serum obtained by injecting
5 New Zealand White rabbits (Pel Freez) with purified mutS
protein. Antibodies were partially purified by protein
G - sepharose (Pharmacia) column chromatography. In dot
blot analysis, 10μg/ml of anti-mutS antibody was found to
detect 1ng purified mutS protein.

10 RESULTS
Detection of mismatches in heteroduplexes annealed in
solution.
    The data presented in Figure 4 indicates that G:T and
G:G containing heteroduplexes can be detected by mutS
15 protein. The detection depends on mutS and can be
competed away with heteroduplex, but not homoduplex, DNA.
Similar results were obtained with A:C mismatches and
heteroduplexes with one unpaired base (data not shown).

Annealing to immobilized oligonucleotides.
20   "Tailed" oligonucleotides were spotted on filters and
30mer oligonucleotides annealed to them. The results
(Figure 5) are identical to those obtained with
heteroduplexes prepared by annealing in solution (Figure
4). Homoduplexes (formed by using 30mer oligonucleotides
25 exactly complementary to the 30mer portion of the
immobilized oligonucleotides) were not detected.

Annealing in the presence of competing oligonucleotides.
    Homoduplexes were prepared by annealing complementary
30mers. "Tailed" oligonucleotides were then added, the
30 homoduplexes denatured and the mixture reannealed.
Samples were spotted on filters. Figure 6 shows that G:T
mismatches are detected under such conditions, indicating

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that the "tailed" oligonucleotides effectively compete
with 30mers for complementary oligonucleotides. Such
conditions parallel those that could be used in a mutation
detection assay. In such an assay, the sample DNA could
5   be cut by restriction enzymes (for genomic DNA) or
prepared by PCR. In either case the DNA would be double
stranded and, therefore, contain sequences identical to
and capable of competing with the tailed oligonucleotides
used as probes.

10   Results presented in Figure 4 were obtained from
experiments carried out as follows:
        Heteroduplexes and homoduplexes were prepared by
        annealing equal amounts of oligonucleotides. Since
        the "tailed" oligonucleotides are 55 bases long,
15   there was nearly a 2-fold excess of 30mers,
        increasing the likelihood that all "tailed"
        oligonucleotides would become double stranded. $5\mu g$
        (in $50\mu l$) of each oligonucleotide in TNE (10mM Tris,
        pH 8.0, 0.1M NaCl, 1mM EDTA) were mixed, heated to
20   70°C for 10 minutes, cooled to room temperature for
        30 minutes and chilled on ice. Annealed molecules
        ($1\mu g$ in $2\mu l$) were spotted on 25mm nitrocellulose
        disks (0.45 micron pore size, Scleicher and Schuell).
        10ng of purified mutS was spotted on the filters as a
25   positive control for the primary (anti-mutS) and
        secondary antibodies and the alkaline phosphatase
        development system. Filters were air dried and
        transferred to 6 well tissue culture plates (Falcon)
        containing 2ml reaction buffer (20mM Tris, pH 7.4,
30   0.01mM EDTA, 0.5mM $MgCl_2$, 0.01mM DTT) + 3% (w/v) non-
        fat dry milk and incubated overnight at 4°C with
        gentle shaking. Filters were washed with cold
        reaction buffer (4x2ml) and incubated (30 minutes at
        4°C) with single strand binding protein (Promega)
35   solution (1ml at $100\mu g/ml$ in reaction buffer).

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Filters were washed with cold reaction buffer (4x2ml) and incubated (30 minutes at 4°C) with (i) 1ml reaction buffer, (ii) 1ml reaction buffer + 10µg/ml mutS or (iii) 1ml reaction buffer + 10µg/ml mutS + 1µg/ml homoduplex or heteroduplex 30mers, annealed as described above. Filters were washed with cold reaction buffer (4x2ml) and incubated (2 hours at 4°C) with anti-mutS antibodies (2ml at 50µg/ml in reaction buffer + 3% non-fat dry milk). Filters were washed with cold reaction buffer (4x2ml) and incubated (1 hour at 4°C) with 2ml of a 1:1000 dilution (in reaction buffer +3% non-fat dry milk of goat anti-rabbit IgC conjugated to alkaline phosphatase (Bio-Rad). Filters were washed with cold reaction buffer (4x2ml) and incubated (10-20 minutes at room temperature) with 2ml fresh development buffer (100mM Tris, pH 9.5, 100mM NaCl, 5mM MgCl$_2$, .165mg/ml Bromochloroindoyl phosphate (diluted 1:100 from stock solution in dimethylformamide), 0.33 mg/ml Nitro-blue tetrazolium (diluted 1:100 from stock solution in dimethylformamide)). Filters were washed several times with water to stop the reaction.

Results presented in Figure 5 were obtained from experiments carried out as follows:

1µg (2µ) of "tailed" oligonucleotides were spotted on nitrocellulose filters. The filters were air dried and transferred to 6 well plates containing 2ml of TNE. 2µg (4µl) of 30mer oligonucleotides were added. The plates were floated in a 70°C water bath for 10 minutes, incubated at room temperature for 30 minutes and placed on ice. Filters were then treated as described above in relation to results presented in Figure 4.

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.Results presented in Figure 6 were obtained from experiments carried out as follows:

Equal amounts of 30mer oligonucleotides were annealed as described in the legend to Figure 4. An equal
5   number of "tailed" oligonucleotides were added and the mixtures heated to 85°C for 10 minutes followed by incubation at room temperature for 30 minutes. All subsequent steps were as described in the legend to Figure 4.

10   It should be pointed out that recA protein can be used in lieu of the single-strand DNA binding protein, in order to facilitate annealing.

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TABLE 2

OLIGONUCLEOTIDE SEQUENCES

1) *ccc...ccGCACCTGACTCCTGGGGAGAAGTCTGCCGT   Mutant[1]

2) *ccc...ccGCACCTGACTCCTGAGGAGAAGTCTGCCGT   Wild Type

3)   CGTGGACTGAGGACTCCTCTTCAGACGGCA   Wild Type

4)   CGTGGACTGAGGACGCCTCTTCAGACGGCA   Mutant

5)   CGTGGACTGAGGACCCCTCTTCAGACGGCA   Mutant

6)   CGTGGACTGAGGACCCCCTCTTCAGACGGCA   Mutant
                                              (+1 Base)

* - 5' aminomodifier
1 - Also prepared without poly C tail and 5'
    aminomodifier

Legend to Table 2
Oligonucleotide Annealings   Sequence ID Number
1 + 3 = G:T Mismatch   1)   SEQ ID #1
1 + 4 = G:G Mismatch   2)   SEQ ID #2
1 + 5 = Homoduplex   3)   SEQ ID #3
1 + 6 = Addition/Deletion Heteroduplex   4)   SEQ ID #4
2 + 3 = Homoduplex   5)   SEQ ID #5
2 + 5 = A:C Mismatch   6)   SEQ ID #6

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EQUIVALENTS

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters,
5   concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

While this invention has been described in connection with specific embodiments thereof, it will be understood
10   that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the inventions following, in general, the principles of the invention and including such departures from the present disclosure as come within known or
15   customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: Wagner, Jr., Robert
                  Radman, Miroslav

   (ii) TITLE OF INVENTION: Method for Detection of Mutations

  (iii) NUMBER OF SEQUENCES: 6

   (iv) CORRESPONDENCE ADDRESS:
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        (F) ZIP: 02173

    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

   (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER: US
        (B) FILING DATE:
        (C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
        (A) NAME: Granahan, Patricia
        (B) REGISTRATION NUMBER: 32,227
        (C) REFERENCE/DOCKET NUMBER: UBI91-01A

   (ix) TELECOMMUNICATION INFORMATION:
        (A) TELEPHONE: 617-861-6240
        (B) TELEFAX: 617-861-9540

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(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 35 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCCCCGCACC TGACTCCTGG GGAGAAGTCT GCCGT   35

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 35 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCCCCGCACC TGACTCCTGA GGAGAAGTCT GCCGT   35

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CGTGGACTGA GGACTCCTCT TCAGACGGCA   30

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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CGTGGACTGA GGACGCCTCT TCAGACGGCA   30

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGTGGACTGA GGACCCCTCT TCAGACGGCA   30

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 31 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGTGGACTGA GGACCCCCTC TTCAGACGGC A   31

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WHAT IS CLAIMED IS:

1. A method for detecting a mutation from a non-mutated
    sequence of a single stranded target polynucleotide
    in a sample, comprising:
5   (a) incubating the sample with a single-stranded
             polynucleotide hybridization partner comprising
             at least one single stranded base sequence which
             is complementary to the non-mutated sequence of
             the target polynucleotide, under conditions
10   suitable to cause hybridization of said
             hybridization partner to any mutated or non-
             mutated sequences of said target polynucleotide
             which may be present in the sample to form a
             hybrid;
15   (b) contacting the hybrid formed in step (a) with a
             mismatch-binding protein; and
        (c) detecting the presence of any mismatch-binding
             protein bound to said hybrid,
        whereby the detection of the presence of the mis-
20   match-binding protein is indicative of the presence
        of a mutation in the sequence of the polynucleotide
        of the sample.

2. A method according to Claim 1 wherein said polynu-
    cleotide hybridization partner is DNA.

25 3. A method according to Claim 2 wherein said hybrid-
    ization partner DNA is cDNA.

4. A method according to Claim 1 wherein said target
    polynucleotide is DNA.

5. A method according to Claim 1 wherein said mismatch-
30 binding protein is the MutS protein or a functional
    derivative thereof.

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6.   A method according to Claim 1 wherein said sample is
      a biological specimen or fluid which has been
      subjected to conditions sufficient to release and
      denature any nucleic acid present therein.

5   7.   A method according to Claim 6 wherein said released
      nucleic acid is subjected to a step of restriction
      endonuclease cleavage prior to said denaturation.

8.   A method according to Claim 1 wherein said polynu-
      cleotide hybridization partner is immobilized on a
10   solid support prior to said incubating of step (a).

9.   A method according to Claim 8 wherein said solid
      support is a nitrocellulose membrane.

10.   A method according to Claim 1 wherein said detection
      step comprises addition of a detectably labeled first
15   binding partner capable of binding to said mismatch-
      binding protein.

11.   A method according to Claim 10 wherein said first
      binding partner is an antibody specific for said
      mismatch-binding protein.

20 12.   A method according to Claim 1 wherein said detection
      step comprises addition of a first binding partner
      which is not detectably labeled, said first binding
      partner being capable of binding to said mismatch-
      binding protein, and a second binding partner capable
25   of binding to said first binding partner and
      detecting the presence of said second binding
      partner.

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13. A method according to Claim 12 wherein said second binding partner is an antibody specific for said first binding partner.

14. A method according to Claim 12 wherein said second
5   binding partner is a MutL protein or a functional derivative thereof.

15. A method according to Claim 14 wherein said second binding partner is a fusion protein between MutL and a second protein or peptide capable of being
10   detected.

16. A method according to Claim 15 wherein said second protein is beta-galactosidase.

17. A method according to Claim 1 wherein said mismatch-binding protein of step (b) is a fusion protein fused
15   with a second protein or peptide capable of being detected.

18. A method according to Claim 17 wherein said detection step comprises addition of a second binding partner capable of binding to said second protein or peptide
20   and detecting the presence of said second binding partner.

19. A method according to Claim 18 wherein said second protein is beta-galactosidase.

20. A method according to Claim 17 wherein said second
25   protein is an enzyme capable of catalyzing the generation of a colored reaction product from a chromogenic enzyme substrate and said detection step

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comprises providing said chromogenic substrate and
visualizing said color reaction product.

21. A method for detecting a mutation from a non-mutated
     sequence of a single stranded target mammalian
5   polynucleotide in a sample, comprising:
     (a) incubating the sample with a single-stranded
          polynucleotide hybridization partner comprising
          at least one single stranded base sequence which
          is complementary to the non-mutated sequence of
10   the target mammalian polynucleotide, under
          conditions suitable to cause hybridization of
          said hybridization partner to any mutated or
          non-mutated sequences of said mammalian target
          polynucleotide which may be present in the
15   sample to form a hybrid;
     (b) contacting the hybrid formed in step (a) with a
          mismatch-binding protein; and
     (c) detecting any binding of said mismatch-binding
          protein and said hybrid,
20   whereby the detection of the binding of the mismatch-
     binding protein is indicative of the presence of a
     mutation in the sequence of the mammalian
     polynucleotide of the sample.

22. A kit useful for detecting a mutation from a non-
25   mutated sequence of a target polynucleotide sequence
     in a sample, said kit being adapted to receive
     therein one or more containers, said kit comprising:
     (a) a first container containing a single-stranded
          polynucleotide hybridization partner comprising
30   at least one single stranded base sequence which
          is complementary to the non-mutated sequence of
          the target polynucleotide;

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·(b)   a second container containing a mismatch-binding
        protein; and

(c)   a third container or a plurality of containers
        containing a reagent or reagents capable of

5   detecting the binding of said mismatch-binding
        protein to said hybrid.

23.   A kit according to Claim 22 wherein said mismatch-
        binding protein is MutS or a functional derivative
        thereof.

10 24.   A kit according to Claim 22 wherein said reagent or
        reagents comprise at least a first binding partner
        capable of binding to the mismatch-binding protein.

25.   A kit according to Claim 24 wherein said first
        binding partner is an antibody specific for the
15   mismatch-binding protein.

26.   A kit according to Claim 19 wherein said reagent or
        reagents comprise a first binding partner which is
        not detectably labeled, said first binding partner
        being capable of binding to said mismatch-binding
20   protein, and a second binding partner capable of
        binding to said first binding partner.

27.   A kit according to Claim 26 wherein said second
        binding partner is an antibody specific for said
        first binding partner.

25 28.   A kit according to Claim 26 wherein said first
        binding partner is a MutL protein or a functional
        derivative thereof.

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29. A kit according to Claim 28 wherein said first
    binding partner is a fusion protein between MutL and
    a second protein or peptide capable of being
    detected.

5 30. A kit according to Claim 29 wherein said second
    protein is beta-galactosidase.

31. A kit according to Claim 29 wherein said second
    binding partner is capable of binding to said MutL
    protein or functional derivative.

10 32. A kit according to Claim 22 wherein said hybrid-
    ization partner is immobilized on a solid support.

33. A kit according to Claim 31 wherein said solid
    support is a nitrocellulose membrane.

34. A kit according to Claim 19 wherein said mismatch-
15   binding protein is a fusion protein fused with an
    enzyme and said reagent comprises a chromogenic
    substrate for said enzyme.

Schematic representation of DNA hybrids to be tested for the presence of mutations

(a) Oligonucleotides

(b) c-DNA

TEST DNA (genomic frag-
ments)

Denaturation and annealing

(a)

(b)
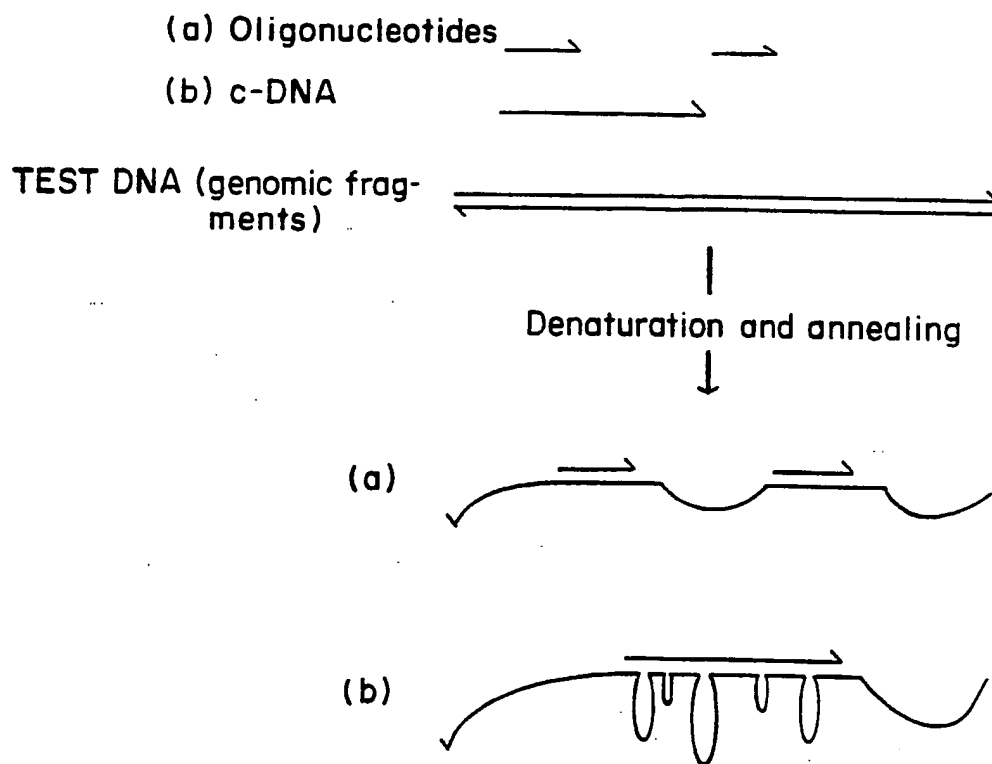
Schematic representation of mismatch-containing DNA hybrids:

(a) Oligonucleotide

(b) c-DNA

FIG. I

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The Principle of Mismatch (Mutation) Detection

Test/Probe DNA hybrids:

Mismatch-binding Protein (MBP)

Anti-Ab

+ Anti-Ab
("amplification")

Ab

Direct detection
via reagents involving
Ab

Detection via reagents involving Anti-Ab
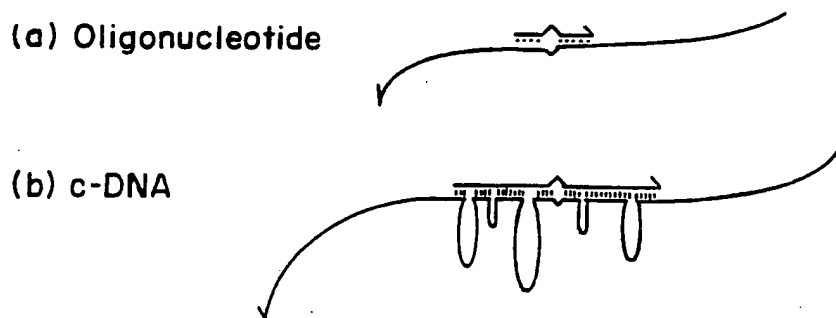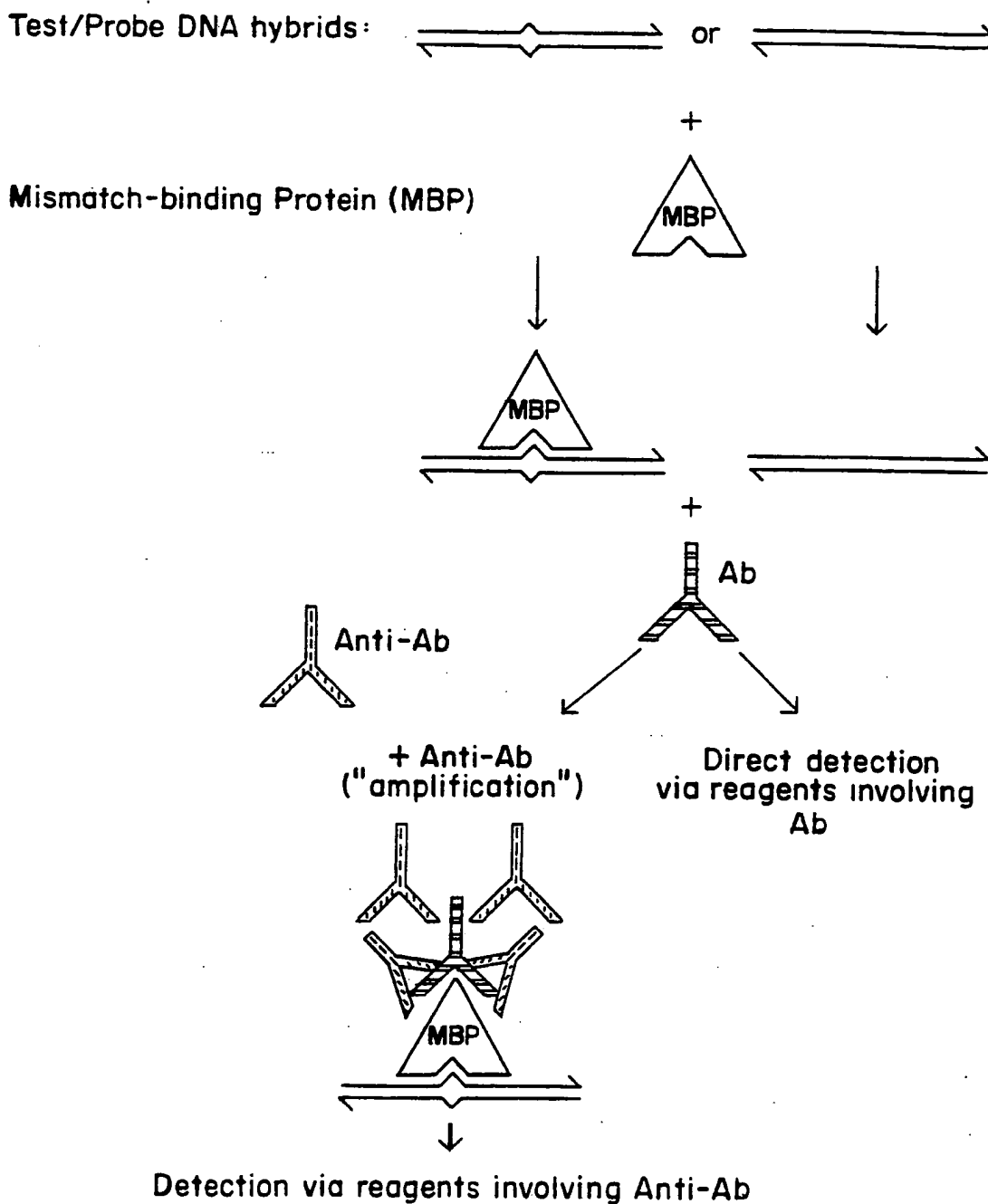
FIG. 2

3/6

Nitrocellulose
Filter with
Probe DNAs
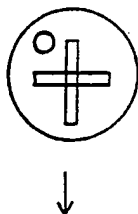
○ normal DNA (negative control)

⊏⊐ mutant DNA sequence (positive control)

⊓ target DNA sequence from normal individuals

① Hybridization with test DNA (or RNA)
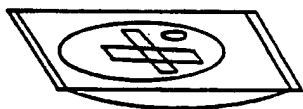
② Filter-blocking treatment

③   + MBP

④   + Anti-MBP (Ab)

⑤   + Anti-Ab

⑥   Reaction with Anti-Ab

⑦   Reading the Result
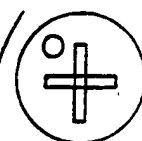
Some of the steps 1-6 did not work.
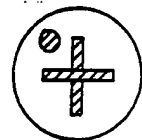
Something is wrong with the system as used.

Correct negative result (no mutation in test DNA).
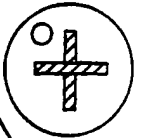
Correct positive result (mutation in test DNA).

FIG. 3

FIG. 4A

G–T

FIG. 4B

G–G

FIG. 5

G–T   G–G   A–C
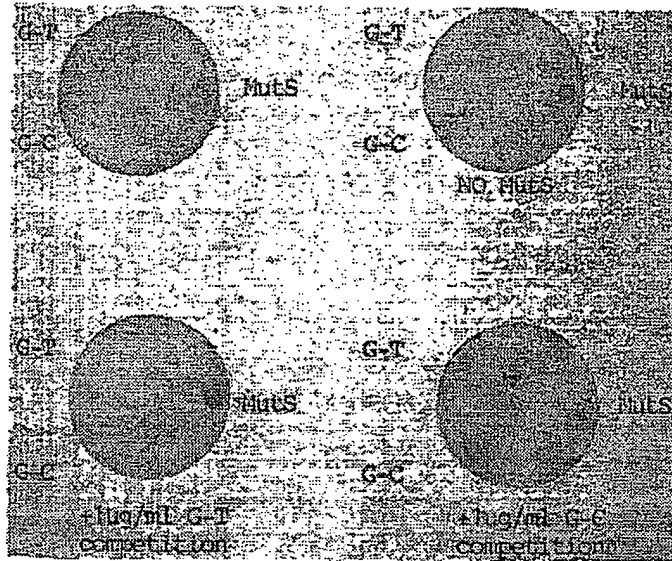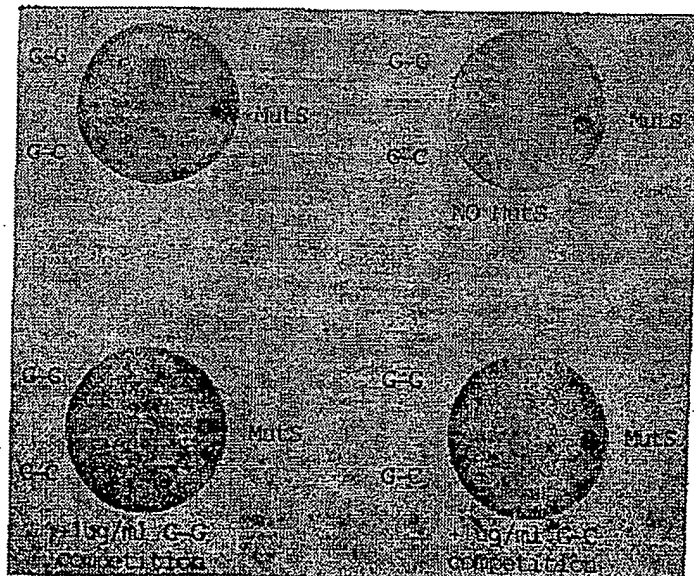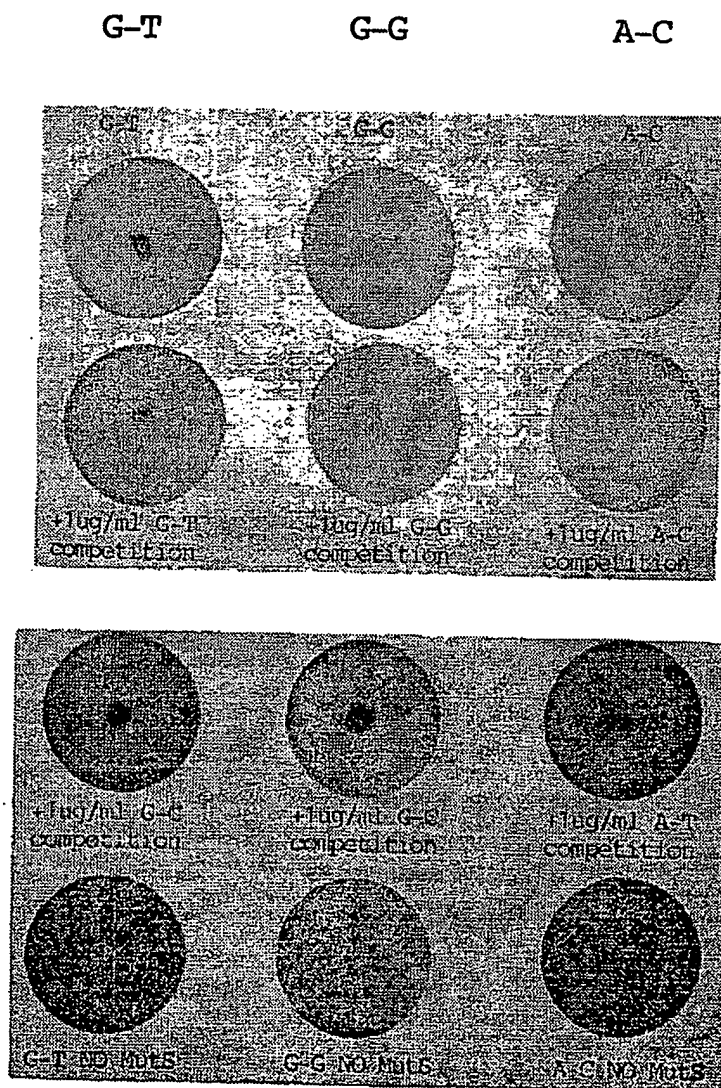
FIG. 6
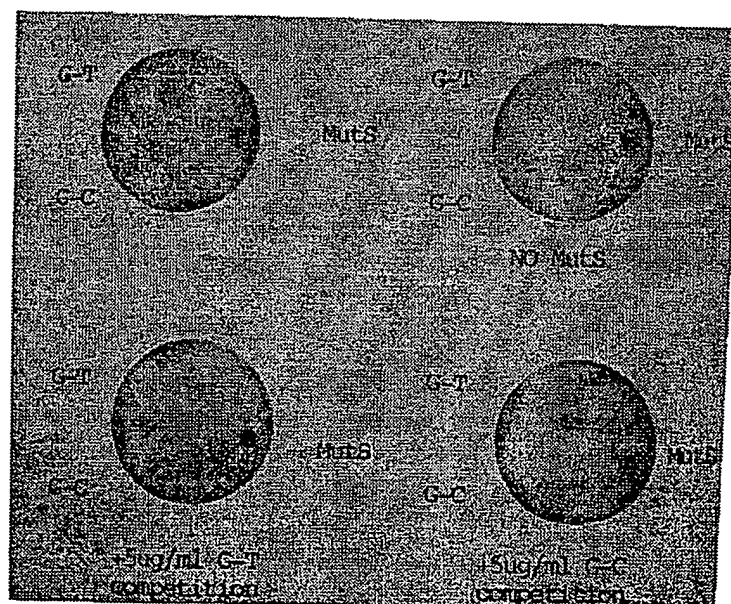
INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/06045

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12Q1/68

II. FIELDS SEARCHED

Minimum Documentation Searched[7]	
Classification System	Classification Symbols
Int.Cl. 5	C12Q

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[o]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
Y	GB,A,2 179 735 (LIFECODES CORPORATION) 11 March 1987 see page 3, line 8 - line 62; claims	1-5
Y	NUCLEIC ACIDS RESEARCH. vol. 16, no. 16, 1988, ARLINGTON, VIRGINIA US pages 7843 - 7853 J.JIRICNY ET AL. 'Mismatch-containing oligonucleotide duplexes bound by the E. coli mutS-encoded protein' cited in the application see the whole document, especially the abstract	1-5

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
11 NOVEMBER 1992	2 1. 12. 92
International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	LUZZATTO E.R.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	EP,A,0 310 251 (MOLECULAR DEVICES CORPORATION) 5 April 1989 cited in the application see page 2, line 40 - line 52; claims	1,6-12

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. US   9206045
SA   62968

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82